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Bacteriocinogeny and Lysogeny in the Genus *Pseudomonas*

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SUMMARY

Bacteriocinogeny and lysogeny have been investigated in strains of *Pseudomonas aeruginosa*, *P. fluorescens* and *P. ovalis*. The 39 strains of *P. aeruginosa* were all bacteriocinogenic and 32 were also lysogenic. No bacteriocine production was detected in 8 strains of *P. fluorescens*, although 3 were lysogenic. The one strain of *P. ovalis* studied was neither bacteriocinogenic nor lysogenic for *P. aeruginosa* or *P. fluorescens*. Typing of the bacteriocines of *P. aeruginosa* on the basis of their action spectra showed that 25 fall into four main groups; the bacteriocines of 6 strains could not be fitted into any definite group.

INTRODUCTION

Bacteriocine production by a strain of *Pseudomonas aeruginosa* (*P. pyocyanea*) was described by Jacob (1954), and investigations into the bacteriocines of 21 Pseudomonas species have since been made by Hamon and co-workers (Hamon, 1956; Hamon, Véron & Péron, 1961). Bacteriocines produced by *P. fluorescens* are known as fluocines; those of *P. aeruginosa* are known as pyocines. The present paper reports on a survey of bacteriocine production and sensitivity among strains belonging to 3 Pseudomonas species. In addition, the activity spectrum of the pyocine produced by each of 31 strains of *P. aeruginosa* has been determined against the other 30 strains in an attempt to establish a rational basis for the classification of the pyocines.

Bacteriocinogeny and lysogeny are both very widespread in the genus *Pseudomonas*, the majority of the strains of *P. aeruginosa* studied by Hamon being either pyocinogenic or lysogenic or both. He also found that, while some of the pyocines were active against several strains of *P. fluorescens* in addition to strains of *P. aeruginosa*, the fluocines produced by strains of *P. fluorescens* were active only against other strains of *P. fluorescens*. Bacteriocine production was rare among the other 19 species examined. The 30 bacteriocines studied in detail were each found to show a different action spectrum towards sensitive strains. The specific resistance or sensitivity of bacterial strains to bacteriocines produced by their own or another species has been noted with many types of bacteriocine (Frédéricq, 1948; Nagy, Alföldi & Ivánovics, 1959; Brubaker & Surgalla, 1961; Hamon & Péron, 1961, 1963) and has been used by Frédéricq (1948, 1953) as the basis for the classification of colicines.

The production of some bacteriocines, like that of certain lysogenic bacteriophages, is inducible by ultraviolet (u.v.) radiation. In some cases (Jacob, 1954; Ivánovics & Alföldi, 1954) lysis of u.v.-irradiated cultures occurs after a characteristic latent

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period, with the release of large quantities of bacteriocine. Lysis is not, however, an obligatory accompaniment of induced bacteriocine production (Ben-Gurion & Hertman, 1958; Hamon & Péron, 1963). The present investigation includes a study of the effect of u.v. irradiation upon bacteriocine production by *P. aeruginosa* strains.

METHODS

Organisms. *Pseudomonas fluorescens* strains A, NS, KB 1, CO 1, CO 2, S 1–A, S 1–B and S 2, and *P. ovalis* Chester were from the culture collection of this laboratory. *P. aeruginosa* strains C 4, C 6 and C 10 were kindly supplied by Dr L. Dickinson; strains 20, 21, 24/1, 24/2, 73, 117, 118, 119, 130 and 149 by Dr M. Rhodes; strains 1, 2, 3 and 29 (as amino acid-requiring mutants) by Dr B. W. Holloway; strains 1999, 2000, 5083, 5940, 6749, 6750, 6751, 7244, 7771, 8058, 8060, 8203, 8505 and 8506 were obtained from the National Collection of Type Cultures. In addition, nine cultures, nominally *P. aeruginosa*, numbered 160, 162, 207, 244, 260, 306, 331, 332 and 333 were isolated in the Department of Bacteriology of this University.

Media. Nutrient broth contained (g./l.) Oxoid tryptone 20, Oxoid Lab Lemco 2: pH 7. Broth cultures of *Pseudomonas aeruginosa* were shaken at 37°, those of *P. fluorescens* and *P. ovalis* at 30°. Peptone agar plates contained *c.* 30 ml. medium comprising (g./l.) Oxoid peptone 10, Oxoid Lab Lemco 1, NaCl 10, New Zealand agar (Northern Media Supply, Ltd., Leeds) 10: pH 7. Soft agar for overlayering contained only 0·6 % (w/v) agar.

Measurement of turbidity of cultures. Irradiated cultures were incubated in conical flasks fitted with side arms into which the cultures could be tipped, enabling turbidity measurements to be made in an EEL nephelometer without sampling.

Irradiation of cultures. Samples (10 ml.) of tryptone broth cultures growing in exponential phase (about 5×10^8 bacteria/ml.) were rocked by hand for 30 sec. in an open 9 cm. Petri dish 1 m. below a Hanovia Chromatolite low-pressure mercury vapour lamp (no filter). After 2-hr incubation, organisms and debris were removed by centrifugation and the supernatant fluids sterilized by heating to 55° for 30 min.

Detection of bacteriocine and phage. The presence of a bacteriocine can be detected by the inhibition of growth of a sensitive indicator organism. Growth inhibition on a seeded indicator plate shows as characteristic clear zones in the area of the applied bacteriocine, and may be indistinguishable from confluent lysis caused by phage. The bacteriocine activity, unlike bacteriophage, cannot be transferred from the inhibition zone to a second indicator plate (Gratia, 1925). The tests used in the present investigation were designed to distinguish activity due to bacteriocine from that due to phage, since many strains produced both bacteriocine and lysogenic phage, and the indicator organism might be sensitive to both agents.

Qualitative tests for the presence of a bacteriocine make use of bacteriocine production on the plate during growth of the producer organism, the producer bacteria being killed before seeding with the indicator. A quantitative estimate of bacteriocine concentration may be obtained by applying to the surface of a seeded indicator plate standard drops of serial dilutions of a cell-free solution of the bacteriocine. The intensity of inhibition of the indicator growth decreases as the bacteriocine concentration decreases, and an end-point can be taken as the highest dilution at

which a zone of inhibition is discernible. This technique also helps to distinguish between inhibition due to bacteriocine and that due to phage, since at high dilutions phage produces discrete plaques on the indicator lawn.

All Pseudomonas strains, except strains 1, 2, 3 and 29 of *Pseudomonas aeruginosa*, were first screened rapidly for bacteriocine and phage production by placing drops of the cell-free supernatant fluid of an overnight culture (undiluted) on seeded indicator plates. The presence of phage was checked by transferring material from each inhibition zone with a platinum wire into 1 ml. tryptone broth, and replating a drop on a second indicator plate. In this way, a qualitative survey of the extent of bacteriocinogeny and lysogeny among the strains was made. Thirty-one strains, selected because they had been characterized by other workers as authentic *Pseudomonas aeruginosa* strains, were exhaustively tested by assaying serial dilutions of the supernatant fluids of u.v.-irradiated cultures. Examination of the inhibition zones revealed the presence of pyocine and/or phage, and gave a quantitative measure of the sensitivity of each indicator to each pyocine. These *P. aeruginosa* strains were also checked by the perpendicular streak method of Abbott & Shannon (1958), a qualitative test involving inhibition by bacteriocine produced during growth of a streak of the producer organism on the plate. This test was not very satisfactory, giving positive results only where the plate assay had already indicated a marked sensitivity to a pyocine. Any reaction (i.e. the result of the action of a given producer on a given indicator) giving a positive result for pyocine in both plate assay and streak test was scored positive, and not investigated further. Reactions showing positive in one test, but negative in the other, were re-tested by the technique of Gratia (1947), which utilizes bacteriocine production by clones of the producer strain growing in the depth of the agar. Only when at least two positive reactions could be obtained from the three tests was the overall reaction scored positive.

Plate assay of culture supernatants. Serial tenfold dilutions (to 10^{-5}) of irradiated and control (unirradiated) cultures of each strain were prepared in tryptone broth, and standard drops of approximately 0·01 ml. were placed on indicator plates seeded with one drop of an overnight culture of the indicator strain. After 16 hr incubation at 37°, the plates were examined. In some cases, complete inhibition of growth occurred even at high dilutions, due to confluent lysis by high concentrations of phage, which obscured the action of any pyocine which might be present. In such cases, the diluted samples were irradiated strongly (10 min. at 25 cm.) to inactivate the phage, and again assayed for pyocine activity. It was assumed that this treatment would not inactivate any pyocine present. Three strains, 8060, 119 and 20, produced phages which were exceptionally resistant to u.v. irradiation, and, as the plaques formed by these phages were exceptionally large (3–5 mm.), confluent lysis occurred when relatively few phage particles were present.

Growth inhibition might also be caused by low molecular weight catabolic products, or by enzymes (extracellular or intracellular) derived from the producer organism. Non-specific inhibition by low molecular weight catabolic products was excluded by assaying dialysed samples of the supernatants of the irradiated cultures. The antibacterial activity of dialysed and non-dialysed samples was the same. A purified preparation of the pyocine of strain c10 was completely inactivated by treatment for 5 min. in a 1 kW. Mullard ultrasonic disintegrator. After similar treatment of the culture supernatant fluids of each strain, the residual antibacterial

activity was negligible. Since enzymes such as the endolysin of *Escherichia coli* can withstand such treatment (Waites & Fry, 1964) it is unlikely that the 'bacteriocine' activity was due to bacteriolytic enzymes of this type.

Streaking test. The procedure of Abbott & Shannon (1958) was followed, the producer organisms being grown on the plates for 2 days before sterilization with chloroform vapour. In addition to the inhibition of indicator growth for 1–2 cm. over the position of the producer streak, characteristic of bacteriocine activity, a second type of reaction, in which growth of the indicator organism occurred over the producer streak but was interrupted for 1–2 mm. on either side, seemed to be associated with bacteriophage action.

Gratia two-layer technique (Gratia, 1947). Plates were seeded with the producer organism so as to give between 20 and 50 colonies per plate. The seeded layer was over-layered with another soft agar layer (2·5 ml.), and the plates incubated 3 days at 37°. The indicator strain was then seeded on to the top of the agar, and examined for growth inhibition after a further 16 hr incubation.

RESULTS

Preliminary screening of all strains by using drops of overnight culture supernatant fluids indicated the presence of many bacteriocine and phage reactions among the various strains of *Pseudomonas aeruginosa* (Fig. 1). None of the *P. fluorescens* strains were bacteriocinogenic, and only one was bacteriocine sensitive; three strains were lysogenic for a phage active against *P. aeruginosa* 5083. *P. ovalis* Chester was inactive in all respects.

All the 31 strains of *Pseudomonas aeruginosa* systematically examined were pyocinogenic and all but 5 were also lysogenic. The pyocine of strain 24/1 was more heat-labile than the others, and it was necessary to sterilize the lysate with chloroform before testing. This strain gave positive reactions only when unheated material was used in the initial screening test. All the pyocines appeared to be u.v.-inducible, different strains showing an increase in pyocine production of between 10- and 1000-fold in the irradiated culture as compared with an unirradiated control. Of the 26 lysogenic strains, 21 showed similar increases in the amount of phage present after irradiation. Strains 6749, 6750 and 6751 showed less phage in the irradiated sample than in the control, titration by plaque count on a suitable indicator giving titres after irradiation of 30, 68 and 60 % respectively of the controls. The phages carried by these strains do not therefore appear to be u.v.-inducible. Strains 5083 and 8203 showed only a twofold increase in phage production after irradiation. It is possible that in all these strains there was interference between bacteriocine and phage production similar to that observed by Frédéricq (1955) and by Kellenberger & Kellenberger (1956), and in consequence the phage yield was reduced.

The systematic cross-testing revealed that the pyocines of many strains could be arranged in groups with similar or identical action spectra, and that strains within each group were generally resistant to pyocines produced by other members of the group (Fig. 2). Since no strain was sensitive to its own pyocine, this would seem to be evidence in favour of the close similarity between the pyocines of the strains in each group, even though in many cases slight differences in spectra were evident. In Fig. 2, areas of cross-resistance between members of each group have been enclosed

in squares for clarity. Four main pyocine groups could be distinguished, into which all but 6 of the strains could be fitted.

A large group, designated group A, contains 13 strains producing pyocins which are active against almost all other strains apart from those within the group. Cross-resistance in group A is not complete, and the pyocines show rather wide quantitative

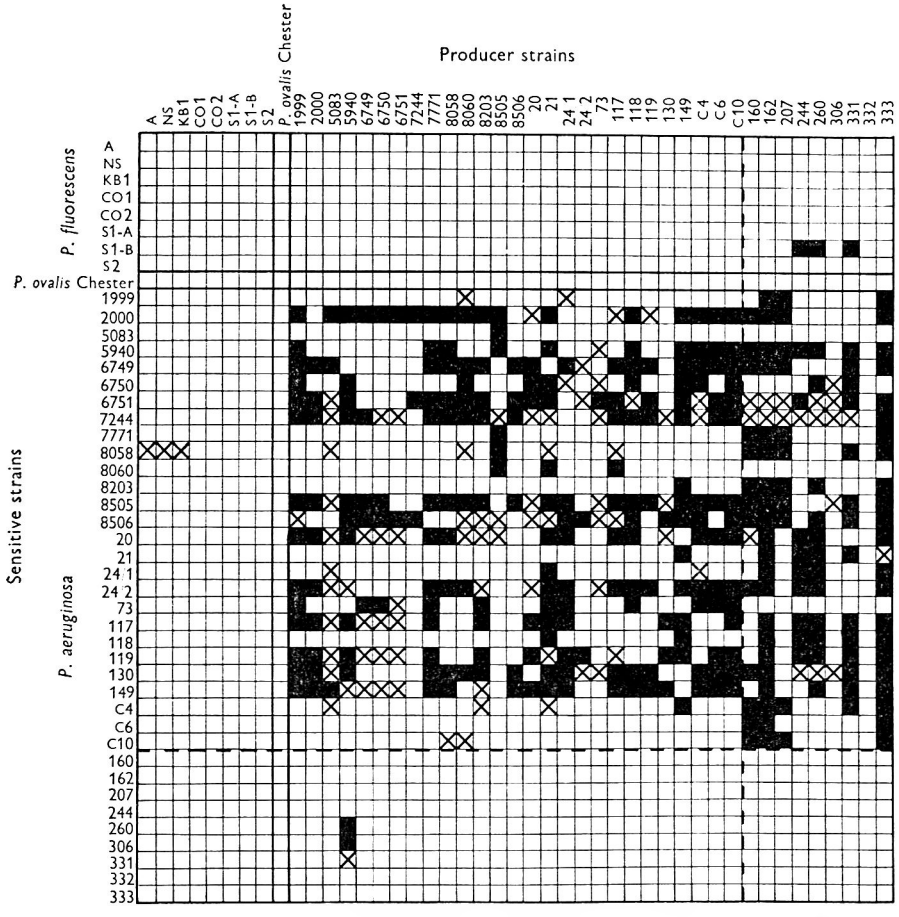

Fig. 1. Bacteriocin and phage interactions among Pseudomonas species. Drops (0·01 ml.) of overnight broth culture supernatant fluids of each strain were tested for their ability to inhibit the growth of every other strain on nutrient agar plates. The inhibition zones were touched with a platinum wire which was washed in 1 ml. broth, and re-tested on a second indicator plate. A solid square denotes inhibition on the first plate only, assumed to be due to bacteriocine action; a cross denotes inhibition on both indicator plates, assumed to be due to phage or to phage plus bacteriocine. Heavy lines divide strains of different species; dotted lines separate the 27 strains of *Pseudomonas aeruginosa* used in the detailed classification of pyocines from those not included.

differences in their spectra, with a graded decrease in activity towards group B strains proceeding from left to right in the diagram. However, it seems difficult to justify subdivision of the group on the basis of action spectra alone. Group B contains 5 strains, with only slightly differing spectra, whose cross-resistance is complete. Group C includes 3 strains with identical spectra and strain 130, which,

although its spectrum differs from the others at four points, has been included on the grounds of its resistance to other members of the group. The pyocines of group C are characterized by their action against strain 5083, and by the large number of strains which are resistant or only slightly sensitive to them. Group D includes 3 strains, 2 of which show only very weak reactions.

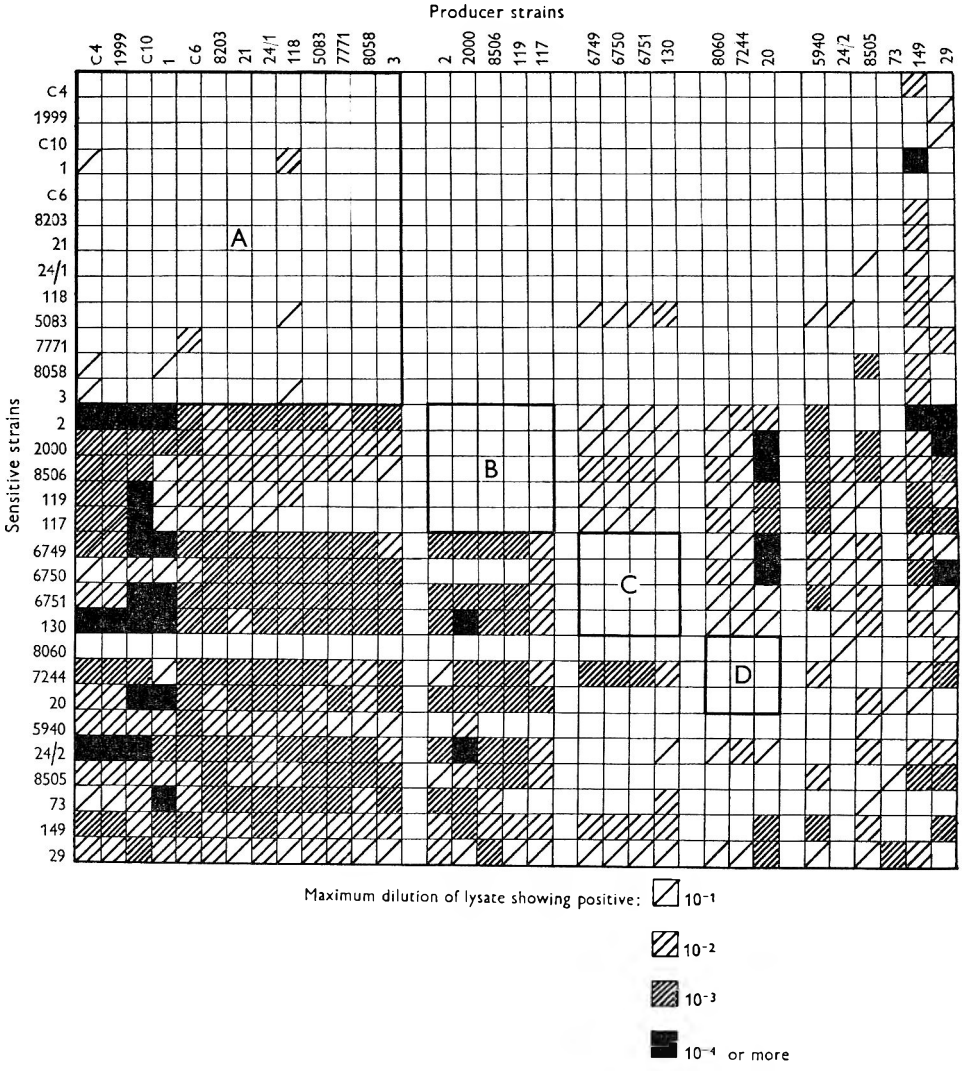

Fig. 2. Action spectra of pyocines from *Pseudomonas aeruginosa*. Each strain was tested for its sensitivity to the pyocine produced by every other strain, by plating serial dilutions of the supernatant fluids of irradiated cultures, and examining for the greatest dilution giving a discernible inhibition zone. Capital letters designate the pyocin type of each group of producer strains.

Of the remaining 6 strains, it is possible that some may show multiple pyocinogeny, since their pyocine spectra overlap with one or more of those within the four main groups. For example, strain 5940 gives a spectrum which overlaps with those of

the pyocines of groups C and D, and moreover is resistant to all but one pyocine in these groups. Strain 149 shows a very wide spectrum, correlating fairly closely with both 8505 and 24/2. However, since the pyocine(s) produced by strain 149 is (are) active against 3 strains which are otherwise pyocine-resistant, and is active against most group A strains, the wide spectrum may indicate only one rather rare pyocine.

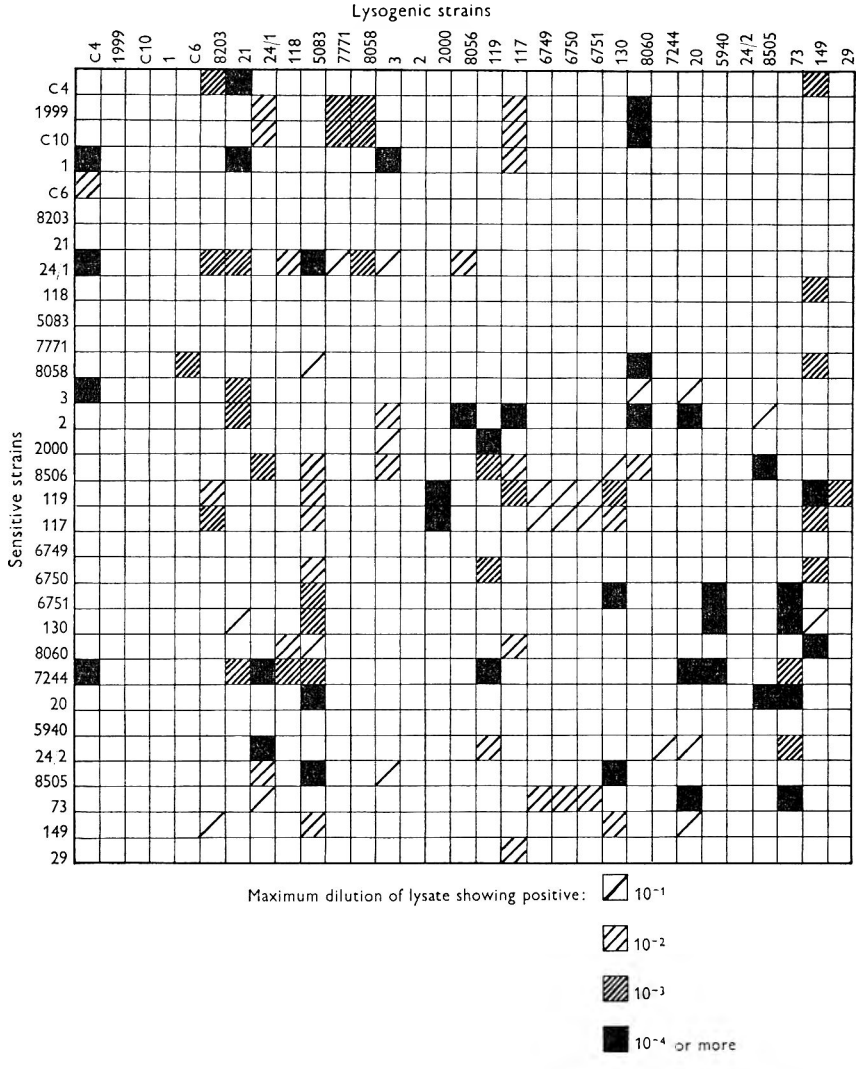

Fig. 3. Action spectra of phages from *Pseudomonas aeruginosa*. Each strain was tested for its sensitivity to the phage produced by every other strain, by plating serial dilutions of the supernatant fluids of irradiated cultures, and examining for the greatest dilution giving plaques in the area of the assay drop.

The spectra of phages from the 31 strains (Fig. 3) do not show the same groupings as the pyocines. The phages have generally very much narrower spectra than the pyocines, and five strains (8203, 21, 5083, 7771, 5940) show complete phage resistance. The considerable overlapping of the phage spectra of different strains indicates that

many may be lysogenic for more than one phage. While there is no general correlation between the phage produced and the pyocine type of any given strain, it is notable that those strains which produce phage with identical spectra also produce pyocines of the same type. Thus strains 7771 and 8058 produce both phage and pyocine with identical spectra, and so also do strains 6749, 6750 and 6751.

DISCUSSION

The results of the above experiments confirm the observations of Hamon and co-workers (Hamon, 1956; Hamon *et al.* 1961) on the very high incidence of bacteriocinogeny and lysogeny in *Pseudomonas aeruginosa*. However, whereas Hamon found that at least three pyocines which showed a wide spectrum of activity against *P. aeruginosa* were also active to some degree against certain strains of *P. fluorescens*, no evidence was found during the present survey for activity against another species of the same genus. Positive reactions against *P. fluorescens* S1–B were given by strains 207, 244 and 331. The identity of these three strains was, however, not established with certainty, and, as they were generally insensitive to the pyocines of authentic *P. aeruginosa* strains, it is doubtful whether they can be classed as typical *P. aeruginosa*. Since the reactions shown among *P. aeruginosa* strains in the initial screening procedure were substantially in agreement with those in the more detailed analysis, it is unlikely that the failure to detect activity against *P. fluorescens* was due to faulty technique. The absence of demonstrable bacteriocinogeny among the strains of *P. fluorescens* examined, in contrast to the 44% incidence reported by Hamon *et al.* (1961), may, however, be due to the relatively small number of strains tested. It is evident from the pyocine spectrum diagram (Fig. 2) that bacteriocines may escape detection unless a large number of possible sensitive strains are used as indicators. Nearly all the strains producing type A pyocines (13 out of 31 studied) are resistant to all but the rarer pyocines, and none of the other strains is sensitive to all the pyocines. General indicators, e.g. strain P10S of Hamon *et al.* (1961), are evidently rare, so that bacteriocine surveys based on the sensitivity of only a few strains, such as those by Holloway (1960), Hamon *et al.* (1961) and Hamon & Péron (1961, 1963) may well be incomplete. The tests used in the present investigation of *P. aeruginosa* strains have shown every strain examined to be pyocinogenic, including Holloway's strain 2, previously reported as non-pyocinogenic (Dr B. W. Holloway, personal communication).

The cross-resistance of individual bacterial mutants each selected for resistance to a particular bacteriocine is a more satisfactory basis for bacteriocine typing than the sensitivity of any naturally occurring strain or strains (Frédéricq, 1953). However, mutation to pyocine resistance seems to be rare, unlike the situation among colicin-sensitive strains, where mutation to colicin resistance is a relatively common occurrence (Frédéricq, 1948), and pyocine-resistant colonies have not so far been observed in this laboratory. Use has therefore been made of the fact that, while all strains are pyocinogenic, none is sensitive to its own pyocine. Pyocine types have been classified according to the resistance not of selected mutants, but of wild-type strains producing a supposedly homologous pyocine. When classified on this basis, however, the pyocines within each group show small heterogeneities in their spectra, and, although this amounts in some cases only to differences in the degree of sensi-

tivity of certain strains, the possibility of subdivision of the main groups arises. Similar small differences in the spectra of many of the colicines investigated by Frédéricq (1948) were later found to be insignificant, and the original 17 types were reduced to 12 by regrouping on the basis of the sensitivities of colicine-resistant mutants. Unequivocal evidence of the identity of the pyocines in each of the major groups (A, B, C, D) described above must await the selection of resistant mutants, the use of antigenic methods, or studies with the purified compounds. The pyocine of *Pseudomonas aeruginosa* C 10 is being purified, and will be reported on in a separate publication. A serological study of the somatic antigens of strains of each pyocine group has shown that the strains of any one group are related antigenically, and differ from those of the other three groups.

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Cycloheximide-producing and Fungicidin-producing Mutants of *Streptomyces noursei*

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SUMMARY

Streptomyces noursei strain 52/152 produced compounds of the cycloheximide series and fungicidin. On the contrary its mutant strain 54/465 did not produce any compound of the cycloheximide series, and as compared with strain 52/152 produced about double the amount of fungicidin. This strain 52/152 was also insensitive to additions to the cultivation medium of fungicidin up to a concentration of 20,000 units/ml. In the strains studied, a direct proportionality was observed between fungicidin production and sensitivity to it. The interrelation of their production is discussed from the viewpoint of a common precursor for fungicidin and cycloheximide (malonyl-coenzyme A).

INTRODUCTION

In a previous paper Spížek *et al.* (1965*a*) studied the influence of mutagenic agents (ultraviolet (u.v.) radiation, X-rays, nitrogen-mustard) on the incidence of biochemical mutants which showed qualitative changes in their pattern of production of secondary metabolites of *Streptomyces noursei*. Spížek *et al.* (1965*b*) showed that fungicidin production could to some extent be influenced by the addition of cycloheximide to the culture medium and conversely that the cycloheximide production could be influenced by additions of fungicidin. In the present paper quantitative correlations between fungicidin and cycloheximide production were studied in selected strains of *S. noursei* (52/152, 54/465, 54/126) and also the sensitivity of these strains to added fungicidin in relation to changes in secondary metabolite production.

METHODS

Mutants 52/152 and 54/465 were industrial strains; mutant 54/126 was a non-producer of cycloheximide and fungicidin. Mutants were isolated after treatment with u.v.-radiation, X-radiation and nitrogen mustard. *Streptomyces noursei*, no. 1475 by selection after treatment with u.v.-radiation, X-radiation and nitrogen-mustard, yielded mutant 52/152; and 52/152 by further similar treatment yielded mutant 54/465, and by X-radiation only mutant 54/126. Ultraviolet irradiation was done with a germicidal lamp (type GE 30 W) at working distance 30 cm.; the time of irradiation (usually 15–30 sec.) was adjusted to yield 0·5–1% survival. X-ray irradiation (Machlett AEG–50T; 50 kV., 30 mA., filter 0·1 mm. Al, 55·040

r./min.) was done with doses 5×10^4r. resulting in about 1% survival. Nitrogen-mustard (*N*-methyl-*β*-dichlorethylamine hydrochloride) was used at 0·01 M in M/15 phosphate buffer (pH 8); after 30 min. treatment there was about 20% survival, and the action of the agent was interrupted by transferring the suspension of conidia to a decontaminating solution (1 g. glycine, 1000 ml. distilled water). The suspensions of conidia (10^6 total conidia/ml.) were mechanically rocked during treatment with mutagenic agents. Viable counts were checked by plating of conidia on cultivation agar medium.

The culture medium used had the following composition (in g./1000 ml.): glucose, 25; Bacto-Peptone, 2; yeast extract, 1; KH_2PO_4, 5; NaCl, 0·5; $MgSO_4.7H_2O$, 0·5; nucleic acid hydrolysate (from yeast nucleic acid), 3; nucleic acid hydrolysate (from thymus nucleic acid), 2; casein hydrolysate, 5; vitamin solution (Hopwood & Sermonti, 1952), 0·1. The solutions of casein and nucleic acid hydrolysates and the vitamins solution were prepared according to Pontecorvo (1953).

Culture medium was distributed in 80 ml. volumes into 500 ml. conical flasks. The medium was sterilized at 125° for 15 min.; final pH was 6·8–7·0.

For inoculation, a conidial suspension (10^6–10^7 conidia/flask) was used and the flasks were incubated for 168 hr on a reciprocal shaker (98 strokes/min., length of stroke 10 cm.).

The appearance of secondary metabolites other than fungicidin was followed by paper chromatography. Chloroform extracts of culture fluid, obtained by separating the mycelium on a centrifuge, were submitted to this analysis. The descending technique was used, on Schleicher–Schüll 2043b paper with the solvent system of benzene + glacial acetic acid + water (6 + 7 + 3, by vol.). The spots were detected by u.v. irradiation (254 mμ, Hanovia Chromatolight) and by the hydroxamate test.

Cycloheximide was assayed by the plate-diffusion method (Growe & Randall, 1955) with *Saccharomyces pastorianus* no. 21–6–1 CHI SAS* as test organism. For the assays of fungicidin, the strain *Candida albicans* no. 44 IEM* was used (Řeháček, 1958).

RESULTS

From the summarized chromatograms shown in Fig. 1 it can be seen that the culture fluid of *Streptomyces noursei* strain 52/152 contained actiphenol in addition to fungicidin and compounds of the cycloheximide series (i.e. cycloheximide, dehydrocycloheximide, anhydrocycloheximide). On the contrary, in strain 54/465 (which produced large amounts of fungicidin) the synthesis of compounds of the cycloheximide series was completely suppressed. This strain, however, did produce a compound with light greenish blue fluorescence, R_F 0·83. Both strains also produced a compound of R_F value 0·70, which showed a yellow fluorescence under u.v. radiation and had growth inhibitory activity against *Bacillus subtilis* 3366 FDA. It can also be seen from Fig. 1 that *S. noursei* strain 54/126 differed from the other strains examined by not producing any of these compounds. It did, however, produce a compound with a violet fluorescence and R_F value 0·73. All three strains produced compounds of the dioxopiperazine type, i.e. 3-isobutylidene-6-benzylidene-2,5-dioxopiperazine (R_F 0·40) and 3,6-dibenzylidene-2,5-dioxopiperazine (R_F 0·93).

* Czechoslovak Collections of Micro-organisms.

In view of our previous finding (Spížek *et al.* 1965*b*), that a close relationship exists between fungicidin and cycloheximide production, it appeared interesting to analyse the interrelation of the production of both antibiotics in the selected strains. It can be seen from Table 1 that fungicidin production by *Streptomyces noursei* strain 54/465 (which only produced fungicidin and no cycloheximide-type substances) was about twice as high as fungicidin production by the original strain 52/152 which produced both fungicidin and compounds of the cycloheximide series.

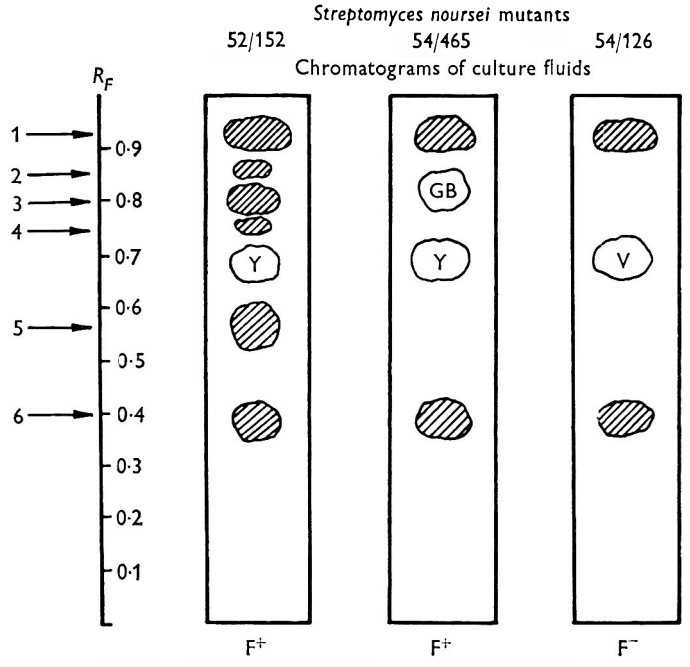

Fig. 1. Chromatographic spectra of products formed during cultivation of *Streptomyces noursei* mutants. V, violet fluorescence (R_F 0·72); Y, yellow fluorescence (R_F 0·70); GB, green-blue fluorescence (R_F 0·83); F^+, presence or F^-, absence, of producing capacity for fungicidin (microbiological plate assay). 1: 3,6-dibenzylidene-2,5-dioxopiperazine; 2: dehydrocycloheximide; 3: actiphenol; 4: anhydrocycloheximide; 5: cycloheximide; 6: 3-isobutylidene-6-benzylidene-2,5-dioxopiperazine.

Table 1. *Relation between capacity for fungicidin production by Streptomyces noursei mutants and resistance to fungicidin concentrations*

Streptomyces noursei strain no.	Production of		Fungicidin resistance (units/ml. cultural fluid)
	Fungicidin (units/ml.)	Cycloheximide (μg./ml.)	
54/465	15,000—	< 0·25	20,000
52/152	6,000	700	2,000
54/126	< 5	< 0·25	20

Data on sensitivity of the individual strains to fungicidin are summarized in Table 1. The high fungicidin producer strain 54/465 was resistant to concentrations up to 20,000 units fungicidin/ml. culture fluid. Strain 52/152 was resistant only to concentrations below 2000 units fungicidin/ml. In contrast to this, the growth of the non-producer of fungicidin strain 54/126 was inhibited even by 20 units fungicidin/ml.

DISCUSSION

In previous papers concerned with cycloheximide biosynthesis (Kharatyan *et al.* 1962; Vaněk, Půža, Cudlín & Doležilová, 1964) it was pointed out that the cycloheximide ring system is built from malonate units (malonyl-coenzyme A). It is likewise evident from studies of fungicidin biogenesis (Rickards *et al.* 1964) that the lactone moiety of the fungicidin molecule is predominantly formed by condensation of malonate units. We consider it therefore very likely that in the case of our *Streptomyces noursei* mutant 54/465 the metabolic pathway leading to compounds

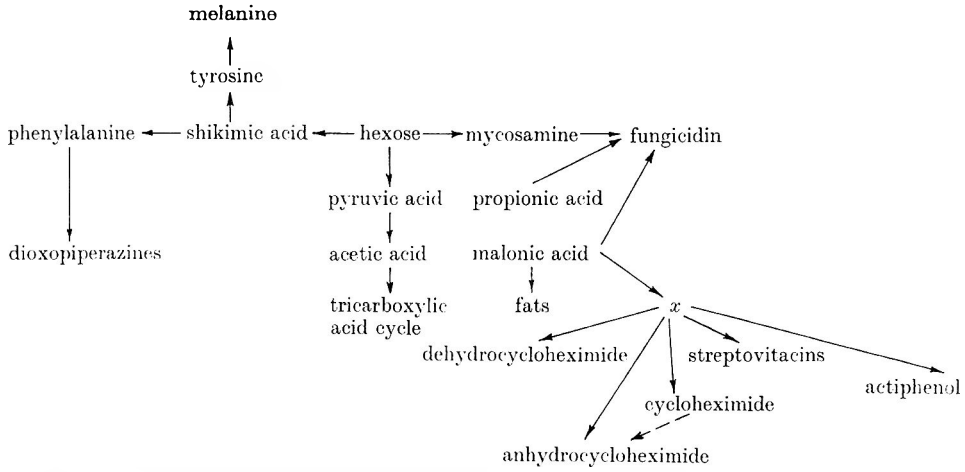

Fig. 2. Scheme of biosynthesis of secondary metabolites of *Streptomyces noursei*. Dotted arrow indicates a possible step not yet proved experimentally.

of the cycloheximide series is blocked, which results in preferential utilization of malonate units for the synthesis of fungicidin. Metabolic pathways leading to secondary metabolites produced by *S. noursei* are schematically shown in Fig. 2. Compounds of the dioxopiperazine type are not directly connected with the biosynthesis of fungicidin and cycloheximide. It follows from the work of Doležilová *et al.* (1965) that phenylalanine is the precursor of these substances (the shikimic acid pathway). When we consider the fact that among biochemical mutants obtained from *S. noursei*, strains can also be found which, while producing no compounds of the cycloheximide series (Spížek *et al.* 1965*a*), did produce fungicidin without showing any increased production capacity for it, we may assume that to obtain an increased ability to produce fungicidin, an additional hit may prove necessary to block a centre responsible for maintaining the high amounts of secondary metabolites produced.

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The Atypical Ribosomal RNA Complement of *Rhodopseudomonas spheroides*

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SUMMARY

Ribosomes and phenol-purified RNA were prepared from *Rhodopseudomonas spheroides* and were characterized by sucrose density-gradient centrifugation and sedimentation-velocity centrifugation. The ribosomes extracted from *R. spheroides* correspond to the 30S, 50S and 70S ribosomes derived from other bacteria. Under appropriate conditions polyribosomes were extracted. The primary ribosomal RNA of *R. spheroides* corresponds to the 16S particle of other bacteria. *Rhodopseudomonas spheroides* was devoid of the 23S ribosomal RNA typically found in other bacteria. Preparations of phenol-purified *R. spheroides* RNA contain, in addition to 16S RNA, smaller amounts of an RNA component which sediments more slowly than the 16S unit, with a sedimentation coefficient of about 14–15S. Of several other Athiorhodaceae examined, only *Rhodopseudomonas capsulata* had an unusual RNA complement like that of *R. spheroides*.

INTRODUCTION

The ribosomal RNA isolated from bacteria consists typically of components with sedimentation coefficients of 16S and 23S. In *Escherichia coli* the 16S particle derives both from 30S and 50S ribosomes; the 23S particle derives only from 50S ribosomes (Kurland, 1960). The 30S and 50S ribosomes aggregate in appropriate magnesium concentrations to form 70S ribosomes which function in protein synthesis.

The studies of Aronson & McCarthy (1961) have suggested that 16S and 23S ribosomal RNAs are built from multiples of a single subunit, the 23S particle being viewed as a dimer of 16S RNA. This view is supported by the work of Spahr & Tissieres (1959), which showed that the 30S and 50S ribosomes of *Escherichia coli* had similar nucleotide compositions, and the work of Petermann & Pavlovec (1963), which indicated that there was interconversion between the 18S and 28S RNA particles of rat liver ribosomes. On the other hand, Aronson (1962, 1963) showed for several bacteria that the RNAs from 30S and 50S ribosomes differed in base sequence. Yankofsky & Spiegelman (1963) reported that 16S and 23S ribosomal RNAs hybridized with different sites on the bacterial genome. These observations imply that 16S and 23S RNA particles are distinct units. The results reported in the present work show that *Rhodopseudomonas spheroides* contains a usual complement

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of ribosomes although it has only one of the usual ribosomal RNA particles, namely that corresponding to the 16S ribosomal RNA of *E. coli*. This suggests that ribosomes can be built up from the 16S unit.

METHODS

Organisms. The strains of *Rhodopseudomonas spheroides* studied were: strain I (NCIB 8253); strain L-81, which was derived from strain L and does not form photopigments; strain Ga, a spontaneous green mutant deriving from strain 2.4.1 of Professor C. B. van Niel's collection; strain M-25, which derived from strain 2.4.7 of Professor van Niel's collection, and requires uracil for growth. Strain M-25 does not grow when orotic acid is substituted for uracil; presumably its pyrimidine synthesis is blocked at some step after the formation of orotic acid.

Rhodopseudomonas spheroides strains Ga and M-25 were obtained from Professor W. R. Sistrom. All other bacteria were obtained from the collection of Dr June Lascelles. Other Athiorhodaceae used were: *Rhodospirillum rubrum* (NCIB 8255); *Rhodopseudomonas palustris*, 2.1.7; *Rhodopseudomonas gelatinosa*, 2.2.13; *Rhodopseudomonas capsulata*, 2.3.11. The last three organisms were originally from Professor van Niel's collection.

The *Escherichia coli* used was laboratory strain 88.

Growth of organisms. All the bacteria were grown to a concentration equivalent to about 100 μg. protein/ml. in the glutamate medium of Lascelles (1959) with succinate substituted for malate (medium SG). For growth of *R. spheroides* strain M-25, the medium was supplemented with uracil (5 μg./ml.).

Escherichia coli was grown aerobically in Erlenmeyer flasks containing volumes of media equal to 1/10 their nominal capacities. The cultures were shaken at 30° on a reciprocal shaker. Aerobic cultures of *R. spheroides* were grown under the same conditions.

Anaerobic cultures of Athiorbodaceae were incubated at 34° in flat medicine bottles or Roux bottles filled to capacity, and were illuminated at the surface of a water bath with a light intensity of about 1200 ft.-c. The cultures were aerated with a gas mixture of 5% (v/v) CO_2 + 95% (v/v) N_2.

Harvesting of cultures. Organisms were centrifuged, washed in 5×10^{-3} M-tris buffer (pH 7·4) containing 10^{-2} M-$MgCl_2$, and then frozen.

Radioactive labelling of bacterial RNA. Rhodopseudomonas spheroides strain M-25 or *Escherichia coli* was grown in SG medium containing per ml. 5 μg. uracil and 0·3 μc. 2-^{14}C uracil (obtained from the Radiochemical Centre, Amersham, Buckinghamshire). The bacteria were centrifuged, washed three times in 5×10^{-3} M-tris buffer (pH 7·4) containing 10^{-2} M-$MgCl_2$, and then frozen.

Estimation of protein and ribonucleic acid. Cell protein was determined by the Folin phenol method (Lowry, Rosebrough, Farn & Randall, 1951) after digestion of organisms in N-NaOH for 2 hr at 40°. RNA was estimated by the orcinol method of Schneider (1945) following extraction from samples with 5% (w/v) trichloroacetic acid for 30 min. at 90°.

Preparation of bacterial extracts and phenol-purified RNA. Frozen organisms were thawed and suspended to a concentration equivalent to between 6 and 15 mg. protein/ml. in 5 ml. of 5×10^{-3} M-tris buffer (pH 7·4) containing 10^{-2} M-$MgCl_2$ and

2 μg. DNAase/ml. (DNAase from Worthington Biochemical Co., New Jersey, U.S.A.). The suspensions were passed through a cold French press under 6000 lb./in.^{2} pressure. The pressed material was incubated for 10 min. at room temperature (about 23°) to allow DNA degradation to proceed. All further steps were carried out at 4°. A portion of the pressed material was centrifuged for 10 min. at 10,000g to remove unbroken organisms and debris. The supernatant fluid was pipetted off, dialysed overnight against 1000 times its volume of 5×10^{-3} M-tris buffer (pH 7·4) containing specified amounts of $MgCl_2$, and used as a source of ribosomes. Under these conditions at least 80 % of the bacterial RNA was recovered in the dialysed supernatant fluid.

The portion of the pressed material left uncentrifuged was shaken with 1/25 its volume of sodium laurylsulphate for 10 min. to dissociate protein from RNA, and the solution deproteinized by the phenol method of Schramm & Gierer (1956). The phenol-purified RNA thus obtained was dialysed overnight against 1000 times its volume of 5×10^{-3} M-tris buffer (pH 7·4) containing specified amounts of $MgCl_2$. Samples of phenol-purified RNA were occasionally frozen before use; this did not alter the sedimentation behaviour of the RNA.

Preparation of polyribosome extracts. Bacteria were suspended to a concentration equivalent to about 50 mg. protein/ml. in 5 ml. of 5×10^{-3} M-tris buffer (pH 7·4) containing 2×10^{-3} M-$MgCl_2$ and 2 μg. DNAase/ml. The bacterial suspension was passed through a cold French press under a pressure of about 1000 lb./in.^{2}, and the pressed material was incubated for 10 min. at room temperature to allow DNA degradation to proceed. All further steps were carried out at 4°. The pressed material was centrifuged for 10 min. at 10,000g. The supernatant fluid was discarded, and the pellet was suspended in 3 ml. of 5×10^{-3} M-tris buffer (pH 7·4) containing 2×10^{-3} M-$MgCl_2$ and 0·3 % (w/v) sodium deoxycholate. After incubation for 5 min. to solubilize the RNA, the suspension was centrifuged for 10 min. at 10,000g. The pellet was discarded, and the supernatant fluid used as a source of polyribosomes. 5–10 % of the bacterial RNA was recovered in the supernatant fluid.

Sucrose gradient centrifugation. Linear sucrose gradients (5 ml.) were prepared in plastic centrifuge tubes appropriate to the SW-39 swinging bucket Spinco rotor by using an apparatus like that described by Britten & Roberts (1960). Portions (0·2 ml.) of crude bacterial extract or phenol-purified RNA followed by 0·1 ml. of sucrose solution (4 or 5 % sucrose for gradients increasing, respectively, from 5 or 15 % sucrose) were drawn into a pipette with a capillary tip. The contents of the pipette were layered on to a sucrose gradient with the same buffer and Mg composition as the RNA sample, and the tubes were centrifuged for specified times at 38,000 rev./min. and 4° in a Spinco model L ultracentrifuge. After centrifugation the tubes were fitted with a stopper attached by tubing to a drawn 10 ml. syringe. The bottoms of the plastic tubes were coated externally with silicone grease and punctured with a small-bore syringe needle. Twenty to thirty 5-drop samples were collected from the gradients by gradually closing the syringe. Portions (0·05 ml.) of each sample were assayed for radioactivity (an Isotopes Development Laboratories liquid scintillation counter was used to measure radioactivity). The remaining fractions were diluted with water, and their absorbancy at 260 mμ was determined.

Analytical ultracentrifugation. Samples of phenol-purified RNA prepared in 5×10^{-3} M-tris buffer (pH 7·4) containing specified amounts of $MgCl_2$ were centrifuged at 20° in a Spinco model E ultracentrifuge. An optical cell with a light path of 12 mm. was used. For schlieren optics the RNA concentration was between 1 and 3 mg./ml., and the bar angle was set between 30 and 60°, depending upon the sensitivity required. For ultraviolet optics the RNA concentration was about 50 μg./ml. The photographic plates obtained from experiments with u.v. optics were analysed by means of a Spinco Analytrol photodensitometer equipped with a microanalyser attachment.

Rhodopseudomonas spheroides RNA, like *Escherichia coli* RNA, sediments more rapidly the higher the magnesium concentration and the smaller the RNA concentration. Since the experiments did not depend upon precise knowledge of sedimentation coefficients, apparent values have been given without correcting to infinite dilution or taking into account the effect of magnesium concentration.

RESULTS

Sucrose-gradient centrifugation

Phenol-purified RNA was prepared from *Escherichia coli* mixed with aerobically or anaerobically grown *Rhodopseudomonas spheroides*, and was characterized by sucrose-gradient centrifugation. The *R. spheroides* RNA included RNA corresponding to the 16S RNA particle of *E. coli* and smaller amounts of RNA which sedimented less rapidly than the 16S unit, but no RNA corresponding to the 23S *E. coli* unit. This is shown in Fig. 1*a* for a mixture of about 95% *E. coli* RNA and 5% ^{14}C-uracil-labelled RNA from *R. spheroides* strain M-25, in 10^{-4} M-$MgCl_2$. The results were the same with RNA obtained from ^{14}C-uracil-labelled *E. coli* combined with excess *R. spheroides*. This observation rules out the possibility that the absence of *R. spheroides* 23S RNA was due to degradation by ribonuclease. In 10^{-2} M-$MgCl_2$, *R. spheroides* RNA tended to aggregate to form RNA broadly sedimenting throughout the 23S region without a well-defined peak (see Fig. 1*b*).

Similar characterization of a mixture of ribosomes from mixtures of *Escherichia coli* and ^{14}C-uracil-labelled *Rhodopseudomonas spheroides* in 10^{-4} M-$MgCl_2$ indicated that *R. spheroides* had a complement of ribosomes corresponding to the 30S and 50S ribosomes of *E. coli* (see Fig. 2*a*). It should be noted that the 50S ribosomes of *R. spheroides* sedimented slightly behind the corresponding *E. coli* ribosomes. In 10^{-2} M-$MgCl_2$, *R. spheroides* ribosomes aggregated to form a ribosomal RNA particle corresponding to the 70S unit of *E. coli* (see Fig. 2*b*). Like the 50S ribosomes, the *R. spheroides* 70S ribosomes sedimented behind the corresponding *E. coli* particles. Furthermore, the *R. spheroides* 70S ribosomes did not dimerize with the more abundant *E. coli* 70S ribosomes to form 100S particles.

Ribosomes sedimenting more rapidly than the 70S units were obtained from *Rhodopseudomonas spheroides* by disrupting the organisms at low pressures in a French press and treating the particulate fraction of the product with sodium deoxycholate to solubilize the RNA particles (see Fig. 3). This class of ribosomes is analogous to the polyribosomal RNA of other investigators (see Schlessinger, 1963). When *R. spheroides* was disrupted at the usual pressures (about 6000 lb./in.^{2}) in 2×10^{-3} M-$MgCl_2$ no ribosomes sedimenting ahead of the 70S units were detected

in either the sodium deoxycholate-treated fraction or the soluble fraction of the pressed material.

Sedimentation-velocity centrifugation

Ultraviolet optics. Resolution of *Rhodopseudomonas spheroides* ribosomal RNA by analytical ultracentrifugation and the use of ultraviolet (u.v.) optics confirmed that *R. spheroides* was devoid of the usual 23S ribosomal RNA. For example, preparations of phenol-purified RNA from *R. spheroides* strain Ga in 10^{-4} M-$MgCl_2$ appeared to contain a single species of ribosomal RNA (see Fig. 4*a*). This RNA corresponded

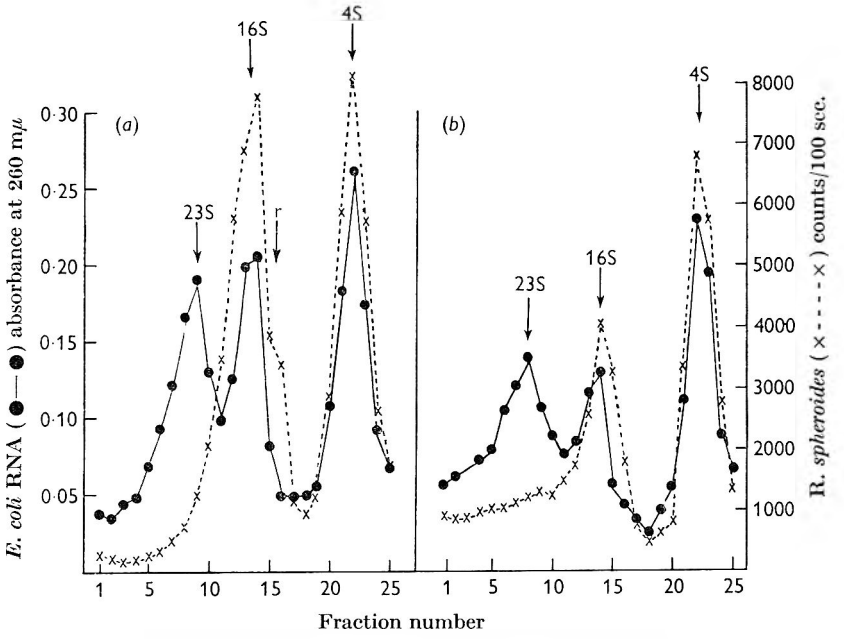

Fig. 1. 2-^{14}C-uracil-labelled organisms from 30 ml. of an anaerobic *Rhodopseudomonas spheroides* culture and unlabelled organisms from 300 ml. *Escherichia coli* culture were treated together to obtain a crude ribosome extract (see Methods). A portion of the ribosome extract was further treated to obtain phenol-purified RNA. One sample of the mixture of *E. coli* and *R. spheroides* phenol-purified RNAs was dialysed overnight against tris buffer containing 10^{-1} M-$MgCl_2$; another sample was dialysed against buffer containing 10^{-2} M-$MgCl_2$. Samples (0·2 ml.) of the RNA dialysed against 10^{-4} M-$MgCl_2$ (about 600 μg. RNA) were centrifuged for 5 hr through linear 5–15 % (w/v) sucrose gradient mixtures containing 10^{-4} M-$MgCl_2$ (Fig. 1*a*). Samples (0·2 ml.) of the RNA dialysed against 10^{-2} M-$MgCl_2$ were centrifuged for 4 hr through 5–20 % (w/v) sucrose gradient solutions containing 10^{-2} M-$MgCl_2$ (Fig 1*b*). Fractions were collected from the gradients. *R. spheroides* RNA (only about 5 % of the RNA applied tc the gradients) was assayed by determining radioactivities of the fractions. *E. coli* RNA was determined by measuring absorbance of the same fractions at 260 mμ.

to the 16S unit of *Escherichia coli* (see Fig. 4*b*). At the same magnesium concentration, 23S RNA comprised a major part of the *E. coli* ribosomal RNA. When *R. spheroides* RNA was prepared in 10^{-2} M-$MgCl_2$ the presence of trace amounts of RNA corresponding to *E. coli* 23S ribosomal RNA was noted (see Fig. 4*c*, *d*). Close examination of the sedimentation patterns of *R. spheroides* RNA revealed a slight

deflexion in the profile of the 16S boundary, indicating a heterogeneity of the '16S' RNA.

Schlieren optics. Characterization of *Rhodopseudomonas spheroides* RNA by analytical ultracentrifugation and schlieren optics confirmed that the occurrence of *R. spheroides* '23S' ribosomal RNA was strictly dependent upon high magnesium concentration (see Figs. 5, 6). In addition, sedimentation analysis with schlieren optics clearly showed the presence of a ribosomal RNA which sedimented less rapidly than did 16S RNA. In contrast, identical analyses of *Escherichia coli* RNA showed that 23S RNA occurred in solutions of low magnesium concentration (see Fig. 5). Furthermore, no particle corresponding to the slowly sedimenting *R. spheroides* ribosomal RNA was observed. We interpret these data to indicate that the basic unit of ribosomal RNA in *R. spheroides* is the 16S particle.

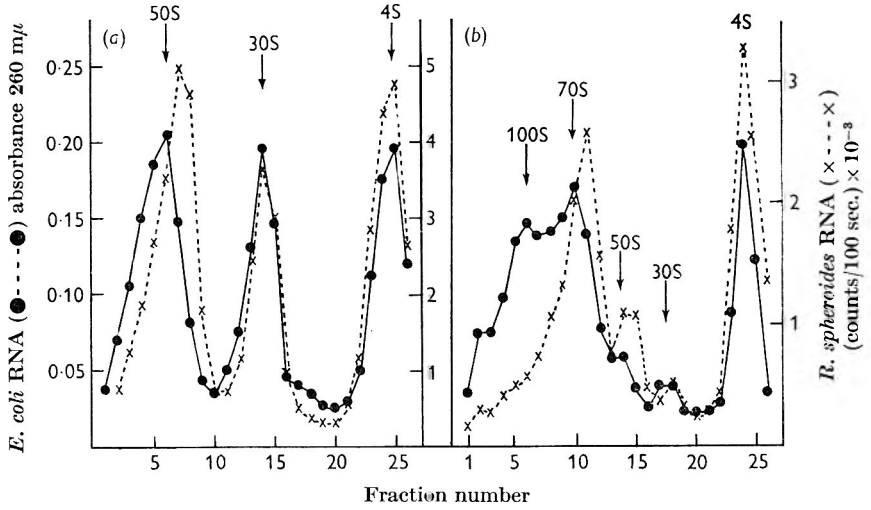

Fig. 2. A portion of the mixture of *Escherichia coli* and *Rhodopseudomonas spheroides* ribosomes described in Fig. 1 was divided into two portions. One was dialysed against tris buffer containing 10^{-4} M-$MgCl_2$ and the other against buffer containing 10^{-2} M-$MgCl_2$. Samples (0·2 ml.) of the ribosome mixture dialysed against 10^{-4} M-$MgCl_2$ were centrifuged for 2·5 hr through 5–20 % (w/v) linear sucrose gradient solutions containing 10^{-4} M-$MgCl_2$ (Fig. 2*a*). Samples of the ribosome mixture dialysed against 10^{-2} M-$MgCl_2$ were centrifuged for 2·5 hr through 15–30 % sucrose gradient solutions containing 10^{-2} M-$MgCl_2$ (Fig. 2*b*). Fractions were collected, and their radioactivities and 260 mμ extinction were measured.

Ribosomal RNA of other Athiorhodaceae

Phenol-purified RNA specimens from *Rhodospirillum rubrum* and the *Rhodopseudomonas* species *R. palustris*, *R. gelatinosa*, and *R. capsulata*, as well as those of *R. spheroides* strains L and L-81, were characterized in 10^{-4} M-$MgCl_2$ by analytical ultracentrifugation and the use of u.v. optics. Only *R. capsulata* and the *R. spheroides* strains L and L-81 had the unusual RNA complement characteristic of *R. spheroides* strains Ga and M-25. The other Athiorhodaceae had a ribosomal RNA complement like that of *Escherichia coli*. Phenol-purified RNA from *R. capsulata* strain 2.3.11

was further characterized by sucrose density-gradient centrifugation and analytical ultracentrifugation with schlieren optics. Both these methods confirmed that *R. capsulata* had a ribosomal RNA complement like that of *R. spheroides*.

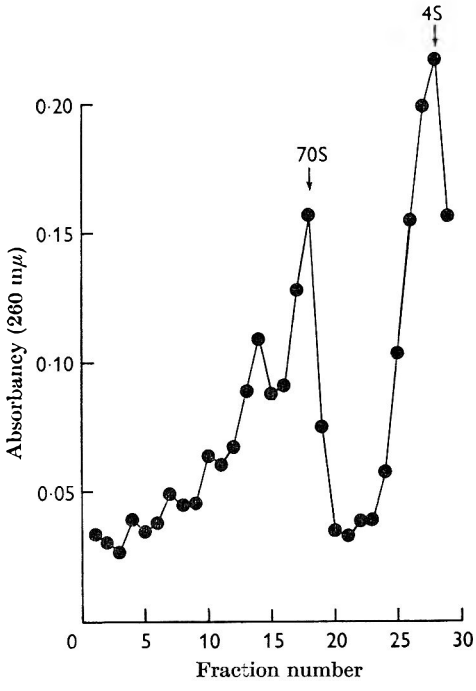

Fig. 3

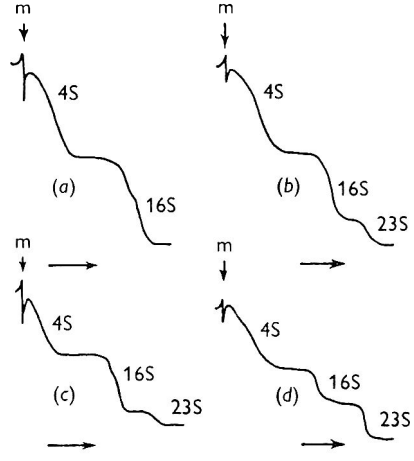

Fig. 4

Fig. 3. An anaerobic culture (900 ml.) of *R. spheroides* strain Ga (see Methods) was poured on to 300 g. crushed ice with 2·4 ml. M-$MgCl_2$ and 6 ml. 2×10^{-1} M-NaN_3. The organisms were centrifuged, and washed in 5×10^{-3} M-tris buffer (pH 7·4) containing 2×10^{-3} M-$MgCl_2$ and 10^{-3} M-NaN_3. The washed bacteria were treated to obtain a polyribosome extract (see Methods). Portions (0·2 ml.) of the extract were applied to 15–30 % (w/v) linear sucrose gradient solutions containing 2×10^{-3} M-$MgCl_2$. The gradient samples were centrifuged for 90 min. at 38,000 rev./min. Fractions were collected and their 260 mμ extinctions determined.

Fig. 4. Organisms from 600 ml. of an anaerobic culture of *Rhodopseudomonas spheroides* were treated to obtain phenol-purified RNA. Phenol-purified RNA was also prepared from 600 ml. of a culture of *Escherichia coli*. One portion of each RNA sample was dialysed against tris buffer containing 10^{-4} M-$MgCl_2$. Another portion of each sample was dialysed against buffer containing 10^{-2} M-$MgCl_2$. The dialysed samples were diluted in buffers containing the same concentrations of $MgCl_2$, to give final RNA concentrations of about 50 μg./ml. The diluted samples were centrifuged at 50,740 rev./min. in an analytical ultracentrifuge fitted for u.v. optics. The sedimentation patterns shown were obtained 24 min. after reaching full speed. (*a*) *R. spheroides* RNA, 10^{-4} M-$MgCl_2$; (*b*) *E. coli* RNA, 10^{-4} M-$MgCl_2$; (*c*) *R. spheroides* RNA, 10^{-2} M-$MgCl_2$; (*d*) *E. coli* RNA, 10^{-2} M-$MgCl_2$. The apparent sedimentation coefficients of the RNA components were as follows: (*a*), 5·5, 17·5; (*b*), 6·6, 16·3, 21·3; (*c*), 4·7, 21·8, 29·6; (*d*) 6·8, 20·6, 31·4. The boundary between the solutions and the air space in the centrifuge cells is indicated by m.

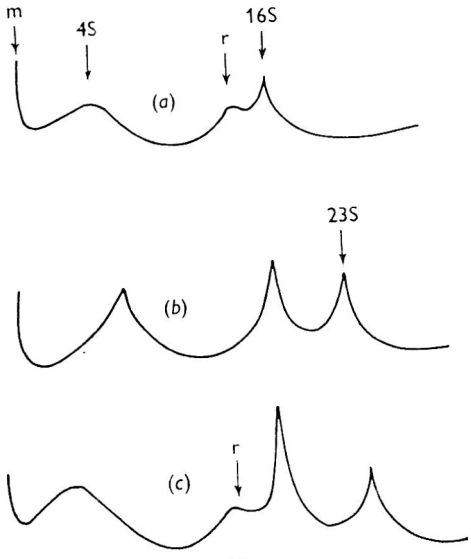

Fig. 5

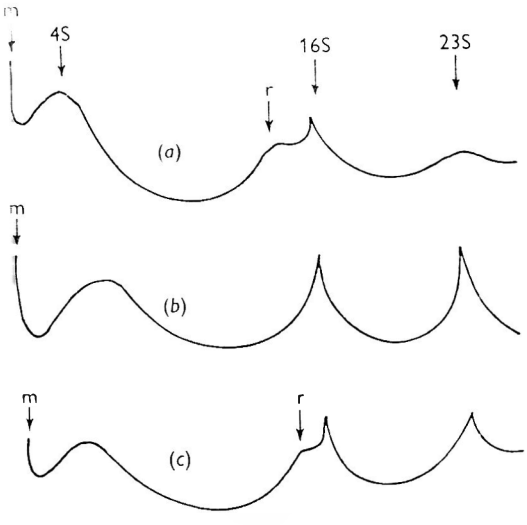

Fig. 6

Fig. 5. The samples of *Rhodopseudomonas spheroides* and *Escherichia coli* used were those described in Fig. 4. Samples which had been dialysed against 10^{-1} M-$MgCl_2$ were diluted in buffer containing the same concentration of $MgCl_2$ to an RNA concentration of about 1·5 mg./ml. A mixture of *E. coli* and *R. spheroides* RNA was obtained by mixing equal volumes of the diluted samples. The mixture and the individual samples were each centrifuged at 50,740 rev./min. in an analytical ultracentrifuge with schlieren optics. The sedimentation patterns shown were obtained 32 min. after attaining full speed. (*a*) R. *spheroides* RNA; (*b*) *E. coli* RNA; (*c*) *R. spheroides* + *E. coli* RNAs. The apparent sedimentation coefficients of the RNA components were: (*a*) 3·7, 10·8, 12·8; (*b*) 5·6, 12·7, 17·2; (*c*) 3·6, 11·8, 14·2, 18·4. The *R. spheroides* RNA which sedimented between soluble RNA and 16S RNA is indicated by r.

Fig. 6. The *Rhodopseudomonas spheroides* and *Escherichia coli* RNA samples used were those described in Fig. 4. Samples which had been dialysed against tris buffer containing 10^{-2} M-$MgCl_2$ were diluted in buffer containing the same concentration of $MgCl_2$ to an RNA concentration of about 1·5 mg./ml. The mixture of *R. spheroides* and *E. coli* RNAs was prepared by mixing equal volumes of each of the diluted samples. The mixture of RNAS and the individual samples were centrifuged at 50,740 rev./min. The sedimentation patterns shown were obtained 24 min. after attaining full speed. (*a*) *R. spheroides* RNA; (*b*) *E. coli* RNA; (*c*) *R. spheroides* + *E. coli* RNAs. The apparent sedimentation coefficients of the RNA components were: (*a*) 3·2, 18·5, 19·6, 29·6; (*b*) 6·5, 19·5, 29·0; (c) 4·0, 17·0, 20·5, 29·0. The *R. spheroides* RNA which sedimented between soluble RNA and 16S RNA is indicated by r.

DISCUSSION

The results presented here strongly suggest that *Rhodopseudomonas spheroides* is able to form its ribosomes from a 16S ribosomal RNA unit. Studies of other bacteria which have suggested that two distinct ribosomal RNAs are necessary to form the usual complement of their ribosomes should be re-examined in the light of the present findings. The work of Petermann & Pavlovec (1963) indicated that there is conversion between the 18S and 28S ribosomal RNA of rat liver. This suggests that the difference between *R. spheroides* ribosomal RNA and that of other micro-organisms may be a difference in the ability of the respective 16S units to dimerize to 23S units. On the other hand, the RNA–DNA hybridization experiments of

Yankofsky & Spiegelman (1963) imply that 23S RNA is not a dimer of 16S RNA, but a distinct unit formed at a different site on the bacterial genome. This view is supported by data which show differences in the base sequences of the RNA from 30S and 50S ribosomes (Aronson, 1962, 1963).

The present observations that *Rhodopseudomonas spheroides* 70S ribosomes did not dimerize with an excess of *Escherichia coli* 70S particles to form 100S units, and that the 50S and 70S ribosomes of *R. spheroides* sedimented behind corresponding *E. coli* particles suggest that the *R. spheroides* 50S ribosome lacks some structural element which is present in the *E. coli* 50S particle. For several reasons it is attractive to speculate that such an element exists, and that it is a low molecular weight RNA. It is consistent with hybridization data, base sequence data, and data which suggest interconversion of 16S and 23S RNA to postulate that there are two classes of 50S ribosomes. One, represented by the 50S ribosome of *R. spheroides*, would be built from two 16S ribosomal RNA units. The other, represented by the *E. coli* 50S ribosome, might be derived from a 23S ribosomal RNA which consisted of two 16S units associated with an additional piece of RNA not present in the *R. spheroides* ribosome. This hypothesis would allow for the interconversion of 16S and 23S RNAs and for the exclusion of 16S RNA from hybridization sites available to 23S RNA.

I cannot offer any explanation for the presence in preparations of *Rhodopseudomonas spheroides* RNA of significant amounts of ribosomal RNA which sedimented more slowly than does 16S RNA. However, Yankofsky & Spiegelman (1963) observed a similar heterogeneity upon salt-gradient elution of *Bacillus megaterium* 16S RNA from methylated serum albumin columns.

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Lysogenic Conversion of Staphylococci to Loss of β-Toxin

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SUMMARY

Staphylococci may produce β-toxin and staphylokinase (fibrinolysin). In clinical material there is a preponderance of strains which are β-toxin negative and kinase positive (β^-K^+), but β^+K^-, β^-K^- and β^+K^+ strains also occur. Strains which are β^+K^- can be converted by lysogenization with certain phages to loss of β-toxin and gain of kinase (β^-K^+). This is true conversion, since every lysogenized coccus carries the new characters. All the phages which produced this double conversion (β^-K^+ phages) belong to the serological group F. The conversion is due to two separate loci on the phage which are active in the prophage state. These loci have not been found in phages of other serological groups (β^0K^0 phages). The β-toxic and fibrinolytic properties of staphylococci are not always phage-dependent; strains which do not carry detectable phages or carry only β^0K^0 phages can be either β^+K^-, β^-K^+, β^+K^+ or β^-K^-, implying the occurrence of structural loci on the bacterial genome. Several strains with the phage-typing pattern 80/81 or a related pattern, which are β^-K^+, were found to carry converting phages. On loss of these phages the strains became β^+K^- and showed a change of typing-pattern, gaining sensitivity for phages of group III.

INTRODUCTION

Lysogenization of certain strains of *Staphylococcus aureus* which produced β-haemolysin with phages belonging to the serological group F resulted in the loss of the ability to form β-toxin (De Waart, Winkler & Grootsen, 1962; De Waart, 1964). This proved to be a true lysogenic conversion (phage conversion) since each lysogenized coccus lost the character and each colony which did not produce β-toxin proved to be lysogenic. More recently a large number of parent strains and lysogenized strains were tested for the production of staphylokinase (fibrinolysin). Conversion to loss of β-toxin was always associated with the gain of staphylokinase. Strains which produced β-toxin but no kinase (β^+K^-) were thus simultaneously converted with regard to two properties into staphylococci of the phenotype β^-K^+. The mechanism of this conversion was studied.

METHODS

Organisms used. All strains of staphylococcus were coagulase positive and typable with the basic set of typing-phages (Blair & Williams, 1961). Apart from the propagating strains of the standard phage-typing set, strain Wood 46 and one

strain isolated from a chicken, all other strains had been isolated from human clinical material in various hospitals.

The strains in phage-group II were described by Winkler & Grootsen (1961). Strain 879 with phage pattern 3B/3C/55/71 was β^+K^- on isolation and could not be shown to be lysogenic in tests with several hundred staphylococcal strains. Strains 756 and 269 with the same phage pattern, and strains 184 and 84 with phage pattern 3A/3B/3C/55/71, had the same properties but were obtained from lysogenic β^-K^+ strains which had lost phage spontaneously.

Strain 57 was sensitive to all phages in groups I and III, and was also β^+K^- and not demonstrably lysogenic in hundreds of tests. Two variants of strain 57 were used; 57 ($\alpha+\beta$) produced α- and β-toxin, 57 β produced mainly β-toxin; many of the experiments were done with the non-lysogenic strains 879, 269 and 57. Some additional strains will be described in the text.

Lysogenic strains will be indicated by the number of the parent strain followed by the number of the phage in parenthesis.

Strains kept well in stab cultures in meat-broth agar at room temperature. Each experiment was started from a single colony on a sheep-blood agar plate derived from the stock culture. Difco nutrient broth and Difco nutrient agar with added $CaCl_2$ (87·6 mg./100 ml.) were used in all experiments.

Phages. Most phages were derived from wild-type strains by induction with ultraviolet (u.v.) radiation (Morse, 1962). The phages in lytic group II described by Winkler & Grootsen (1961) were arranged in three groups, γ, δ and ϵ according to their lytic spectra on fifty strains. The phages within each group are probably co-immune. Group γ and δ are more closely related than γ and ϵ or δ and ϵ. Of the γ phages, 756 and 184 lysed propagating strains (PS) 3B, 3C, 55 and 71, and 780, 84, 246 and 285 lysed PS 3B, 3C and 55. The δ phages (269, 764, 334, 681 and 763) lysed PS3C and 55, and PS3A weakly. The ϵ phages (491, 273 and 509) lysed PS3B and 71.

The phages 3C, 71, 77 and 42D from the basic set were also used. Some phages were isolated from propagating strains of the basic set, e.g. phage L80 from strain PS80, phage L55 from strain PS55, phage L6 from strain PS6, phage L7 from strain PS7. Phage W46 was isolated from strain Wood 46, the well-known α-toxin producing strain.

Phage-typing was done by standard techniques (Blair & Williams, 1961). Phage methods were as described by Adams (1959). Staphylococcal strains were lysogenized by spotting the phages on flooded plates. The secondary growth was suspended in broth and spread on sheep-blood agar plates; β^+ and β^- colonies were easily distinguishable on these plates. Many colonies (from ten to several hundreds depending on the case) were tested for lysogenicity and immunity by cross-streaking with the phage and the indicator strains. In more quantitative experiments about 10^8 phage particles and 10^8 cocci were mixed, incubated for 5 min. and centrifuged. The numbers of surviving cocci and of plaque-forming units were determined in the supernatant fluid and in the sediment. Several hundreds of survivor colonies were scored for β-toxin on sheep-blood agar plates, and for lysogenicity by replicating on indicator plates or by cross-streaking (de Waart *et al.* 1962, table 1).

Toxin production. Toxic filtrates were produced by inoculating 1 ml. of an 8 hr culture into 100 ml. Todd-Hewitt broth in screw-capped bottles of 1 l. capacity

The bottles were then gassed with air + 10 % (v/v) CO_2, closed and shaken at 37°. β-Toxin began to appear at 6 hr, α-toxin several hours later. At 8 hr the culture fluid contained β-toxin without α-toxin; at 16 hr the amount of both toxins was maximal. For routine use, culture fluids were harvested at 16 hr.

Crude culture filtrates and more purified preparations precipitated with 60 % saturated $(NH_4)_2SO_4$ were titrated for β-toxin and α-toxin by incubation of serial dilutions with a 2 % (v/v) suspension of washed red cells from sheep or rabbit in veronal buffer for 1 hr at 37° and 4 hr at 4°. Haemolysis (100 %) was read visually or 50 % haemolysis was determined with an EEL colorimeter. Antitoxins were prepared by injecting rabbits with formalinized α-toxin and β-toxin adsorbed on $AlPO_4$.

Production of anti-β-toxin. Since antisera containing anti-β-toxin but no anti-α-toxin seem difficult to obtain, the production of anti-β-toxin may be described) Staphylococcus strain 269 was cultured in Todd-Hewitt broth under 10 % (v/v) CO_2 as described. The fluid was harvested after 7 hr and centrifuged. (Since it is now known that strain 269 does not produce α-toxin 16 hr cultures may also be used.) The combined supernatant fluids from 10 bottles had a titre of 50 for sheep red cells, whereas the titre for rabbit cells was negligible (< 1). 1350 ml. of a saturated solution of ammonium sulphate (Merck p.a.) were added to each litre of supernatant fluid and the mixture kept at 4° for 16 hr. After centrifugation the sediment was dissolved in 0·15 M-phosphate buffer (pH 6·8) and dialysed in a cellophan (Nojax) bag against 300 ml. buffer for 3 hr on a magnetic stirrer, the buffer being replaced each hour. The resulting brown fluid had a titre for sheep red cells of 1000. The titre for rabbit cells was < 1.

Toxoid was prepared by adding 1 ml. of a 10 % (w/v) solution of formaldehyde to 10 ml. of toxin solution and incubating for 3–4 days at 37°. The titre for sheep red cells was decreased to below 1. Excess of formaldehyde was eliminated by dialysis against buffer. The toxoid was brought to pH 5·4 with 5 % (w/v) HCl and one volume of a suspension of 25 mg. $AlPO_4$/ml. was added. The mixture was incubated overnight at 37° and kept at 4°. Rabbits were injected subcutaneously in the abdominal wall on days 1, 3, 5 and 7 with respectively 1, 3, 5 and 7 ml. of this toxoid. Ten days later serum was obtained and tested for antitoxin. The amount of serum that neutralized 100 50 % haemolytic doses (HD 50) was taken as a unit of antitoxin (AU). Sera of about 50 AU/ml. were obtained.

Staphylokinase. Kinase was demonstrated by the production of a clear zone on nutrient agar plates containing heated (20 min., 56°) rabbit plasma (Lack & Wailling 1954). Soya bean trypsin inhibitor did not decrease the zone. No zones were produced on fibrinogen without plasminogen. Culture filtrates containing kinase were produced by shaking cultures of 50 ml. Todd–Hewitt broth in screw-capped bottles at 30°, with air as gas phase. Precipitation with 4 vol. of ethanol (Gerheim, Ferguson & Travis, 1947, 1948; Gerheim, 1948; Gerheim, Ferguson, Travis, Johnston & Boyles, 1948) produced an appreciable concentration without loss of activity. A purification by the method of Glanville (1963) was confirmed but was not used for the work reported in this paper. A rough assay by a cup-plate method on agar plates with heated rabbit plasma in Michaelis buffer (pH 7) was used for testing. The amounts of kinase were expressed in μg. of a crude (standard) preparation.

RESULTS

Simultaneous conversion to loss of β-toxin and to kinase production

Staphylococcus strain 879 produces α-toxin and β-toxin but no kinase. After lysogenization with some of the γ, δ and ε phages described under Methods, the lysogenized strains had lost the β-haemolytic character as tested on sheep blood agar and produced kinase as tested on heated rabbit-plasma agar. The haemolytic activity of culture filtrates of the lysogenized strains on sheep red cells decreased by a factor 10 or more. A selection of results is presented in Table 1. The conversion of strain 879 from β^+K^- to β^-K^+ was produced by the first seven phages shown. It was also produced by phages 184, 780, 764, 334, 681 and 763 which have been omitted from the table. The conversion was not produced by the three phages of serological groups A and B or by phage 509 which also belongs to group F.

Table 1. *Influence of lysogenization on the production of β-toxin and staphylokinase by Staphylococcus strains*

R and S = reciprocal of dilution of standard culture filtrate giving 50 % haemolysis with rabbits cells and sheep cells, respectively. r = ratio of S of parental and lysogenized strain. K = kinase test on heated rabbit plasma.

Strain no.	Origin of phage*	Serological group of phage	R	S	r	K
Strains in phage group II						
879	.	.	86	811	.	−
879 (756)	γ	F	274	46	17	+
879 (84)	γ	F	370	76	10	+
879 (246)	γ	F	340	69	11	+
879 (285)	γ	F	490	69	11	+
879 (269)	δ	F	354	40	20	+
879 (273)	ε	F	443	69	11	+
879 (491)	ε	F	352	82	9	+
879 (509)	ε	F	42	877	0·9	−
879 (L55)	PS55	A	172	2590	0·3	−
879 (71)	St	B	276	1150	0·7	−
879 (3C)	St	A	170	1390	0·6	−
269	.	.	74	1600	.	−
269 (269)	δ	F	71	49	33	+
Strains in phage groups I and III						
57α+β	.	.	575	2030	.	−
57α+β (756)	γ	F	511	59	34	+
57β	.	.	34	304	.	−
57β (42D)	St	F	81	6	50	+
57β (L80)	PS 80	F	51	3	101	+
57β (W46)	Wood 46	F	30	3	101	+
57β (77)	St	F	.	200	.	−

* γ, δ, ε = phages described by Winkler & Grootsen; St = phages from standard phage-typing set; PS55, PS80, Wood 46 indicate that the phages were isolated from these strains.

The experiment was repeated with Staphylococcus strain 184 which was again converted by the F phages 184, 756, 285 and 269, but not by the A phage L55 or the B phage 71 (not in Table 1). With strain 269 analogous results were obtained.

Consequently 13 out of 14 of the group F phages of lytic group II gave the conversion of both characters. Since the phages within each of the γ, δ and ϵ groups are co-immune, these 13 phages probably represent only three unrelated instances of the phenomenon. It was shown earlier that the change of properties was due to true conversion. It should perhaps be added that occasional β-haemolytic colonies which were non-lysogenic could be isolated from the lysogenized strains, showing that the conversion was reversible.

In the hope of extending the material outside Staphylococcus strains of group II, strain 57, which is β^+K^- and sensitive to all phages of lytic groups I and III, was used to test the basic set of phages and over 50 phages from natural strains. Only three further instances of double conversion were found as shown in the second part of Table 1. The three converting phages proved to belong to the serological group F. However, the F phage 77 did not convert. The serological type of all the non-converting phages was not studied, but strain 57 was not converted by phages 6, 7 and 81 from the basic set which belong to serological group A. These results show that some phages are able to convert β^+K^- strains to β^-K^+. They seem to occur only in serological group F, though not all group F phages carry this property.

By neutralization experiments with anti-α and anti-β-toxin (Table 2) it was shown that β-toxin production was absent from the converted strains and that the residual haemolysis of sheep red cells was due to other toxins (α- toxin, δ-toxin).

Table 2. *Neutralization of haemolysins of a parental and a lysogenized strain of Staphylococcus by pure anti-α-toxin and anti-β-toxin*

R and S are reciprocals of 50% haemolysis titre against rabbit and sheep red cells, respectively.

	R	S
Strain 879		
Culture filtrate	70	937
+ normal serum	63	660
+ 1·8 units anti-α-serum	1	731
+ 2 units anti-β-serum	79	2
Strain 879 (756)		
Culture filtrate	296	53
+ normal serum	276	22
+ 1 unit anti-α-serum	2	0
+ 2 units anti-β-serum	610	15
Strain 57 α + β		
Culture filtrate	624	1500
+ 1·8 units anti-α-serum	2	1380
+ 2 units anti-β-serum	650	69
Strain 57 α + β (756)		
Culture filtrate	648	82
+ 1·8 units anti-α-serum	2	15
+ 2 units anti-β-serum	661	51

Independent character of β-toxin and staphylokinase

Christie & Wilson (1941) and Rountree (1947) noticed that β-toxin and kinase were not often produced by the same Staphylococcus strain or, in other words, that β^+K^- strains and β^-K^+ strains were frequent and β^-K^- or β^+K^+ strains rare. Analogous observations were made by Willis, Jacobs & Goodburn (1963). The

possibility that kinase broke down the β-toxin or interfered with its action, or that β-toxin broke down or interfered with kinase, was excluded by incubating preparations of both enzymes together and subsequently measuring their activity (Table 3). Since the activity of both enzymes was found to be unimpaired, inactivation or interference was not the cause of the observed correlation. Since purified kinase is not haemolytic (Glanville, 1963) and kinase preparations are not neutralized by anti-β-toxin (Table 3), kinase and β-toxin can be considered as separate proteins.

Table 3. *Absence of interaction between kinase and β-toxin*

A concentrated crude staphylokinase preparation (20 mg./ml.) and a staphylococcal culture filtrate containing β-toxin (2000 HD50/ml.) were mixed with each other or with broth. Kinase was also tested with anti-β-toxin. After incubation at 37° for 1 hr the fluids were titrated for β-toxin and/or kinase.

Staphylo-kinase (ml.)	Staphylo-coccus β-toxin (ml.)	Broth (ml.)	Anti-β toxin (ml.)	Staphylo-kinase (mg.)	Staphylococcus β-toxin HD50 doses
1	.	1	.	20	.
1	1	.	.	30	2000
.	1	1	.	.	2000
1	.	.	1	25	.

Strains with various combinations of β-toxin and kinase

Lysogenic conversion of staphylococcal strains from β^+K^- to β^-K^+ and the reversal by loss of phage can be interpreted by considering the lysogenized strains as partial diploids with β^+ and K^- genes on the bacterial genome and dominant β^- and K^+ genes on the prophage. The various combinations of properties in staphylococcal strains might then be caused by variation in the bacterial genome, as well as in the phage genome. It seemed of interest to examine the frequency of phage involvement and of the distribution of types. That many naturally occurring β^-K^+ Staphylococcus strains do not easily yield a converting phage had already become apparent in the lysogenization experiments with strain 57.

Strains with unusual combinations such as β^-K^- and β^+K^+ were sought. Among the propagating strains of the standard set PS6, PS7 and PS73 proved to be β^-K^-. Among 40 naturally occurring strains, selected at random from a collection of 3000 strains, two β^-K^- strains were found, i.e. strain 1835 (isolated 25. iv. 1961 from a nasal swab) with phage pattern 47/70/73/42E and strain 8289 (isolated 15. i. 1962 from a chicken) with pattern 52A/79/80. No true β^+K^+ strain was found in this series. Later strain 891, with phage-pattern 44/70 (29/44B/52/52A/6/7/53/83A weak) was found to be truly β^+K^+. These six strains were tested for lysogenicity against all propagating strains and against strains 879 and 57. Where phages were present, the filtered lysates obtained by induction and purified phages propagated on suitable strains were tested for converting properties. No converting phages were found, though some strains carried non-converting (β^0K^0) phages. This enabled us to classify the six new strains together with the strains in Table 1 in groups according to the β and K character of the bacterial genome, and according to the presence (as far as detected) of converting and non-converting phages. The results are shown in Table 4.

The strains discussed in Table 1 are listed in Table 4 (by a few representatives only) in column C (β^+K^-). The first row contains those that were not demonstrably lysogenic, the second row the strains carrying non-converting (β^0K^0) phages, and the third row the strains containing converting phages.

The five new β^-K^- strains are in column B. Staphylococcus strains PS73 and 1835 are in the first row as they proved to be not demonstrably lysogenic. Staphylococcus strains PS6, PS7 and 8289 are in the second row as they carried non-converting phages. The crude induced phages from strains PS6 and PS7, as well as purified propagated phages from these strains, were used to lysogenize strain 891 (β^+K^+).

Table 4. *Various combinations of the characters β (β-toxin) and K (kinase) in Staphylococcus*

Columns A, B, C and D refer to strains which carry the combination β^+K^+, β^-K^-, β^+K^- and β^-K^+ when not converted (bacterial genotype). The strains in row 1 are non-lysogenic. Those in row 2 carry a non-converting phage (β^0K^0). In these cases the phenotype is identical with the genotype of the staphylococcus. Row 3 contains the strains carrying converting phages (β^-K^+). The strains show the β and K character of the converting phage. The Roman numbers indicate the phage group (Williams & Rippon, 1952) to which the strain belongs.

	A	B	C	D
Row 1. Non-lysogenic	β^+K^+ (−) 891 (III)	β^-K^- (−) PS73 (III) 1835 (I)	β^+K^- (−) 879 (II) 184 (II) 269 (II), etc. 57 (I–III)	β^-K^+ (−) 3A (II)
Row 2. Carrying non-converting phages	β^+K^+ ($\phi\beta^0K^0$) 891 (L6) III 891 (L7) III	β^-K^- ($\phi\beta^0K^0$) PS6 (III) PS7 (III) 8289	β^+K^- ($\phi\beta^0K^0$) 879 (509) II 879 (L55) II 879 (71) II 57 (77) I–III 57 (8289) I–III	β^-K^+ ($\phi\beta^0K^0$) many wild-type staphylococci
Row 3. All strains converted to β^-K^+		β^-K^- ($\phi\beta^-K^+$) PS6 (W46) III PS7 (W46) III PS73 (W46) III	β^+K^- ($\phi\beta^-K^+$) 879 (756) II 184 (184) II 57 (L80) I 57 (W46) I–III	

No β^- colonies were detected among several hundred colonies of the secondary growth, i.e. no conversion was observed. The phage from strain 8289 was used in the same way to lysogenize strain 57; no conversion was observed. (The lysogenized strain 57 (8289) is added in column C, row 2.) These results seem to indicate that strains PS6, PS7 and 8289 did not carry converting phages. That PS6, PS7 and also PS73 did indeed not carry converting phages was proved beyond doubt by lysogenizing them with the converting phage W46: the resulting strains were converted to β^-K^+. Obviously the converting (β^-K^+) phage W46 cannot suppress β-toxin production in a strain which does not produce any. These converted strains are consequently placed in column B, row 3. The β^+K^+ strain 891 was not demonstrably lysogenic and is put in column A, row 1; by lysogenization with the phages L6 and L7 the strain was not converted. These lysogenized strains are put in column A, row 2. In column D only Staphylococcus strain PS3A is cited as an instance of a strain with characters β^-K^+ which was not demonstrably lysogenic.

Many naturally occurring strains which carry β^0K^0 phages would belong in column D, row 2. It has already been mentioned that only a few of them yield converting phages. Whether the β^-K^+ character might be due to a defective converting phage is of course difficult to decide.

Considering these data it is obvious that staphylococcal strains with the characters β^+K^- and β^-K^- and not carrying a converting phage occur in nature. This is probably also true of the characters β^-K^- and β^+K^+, though defective phages of the type β^-K^+ and β^0K^+ cannot be excluded. In any case, it can be concluded that β-toxin and kinase production are separate properties of staphylococci which can be independent of prophage. Furthermore, it is clear from row 3 that until now only converting phages of the type β^-K^+ have been found, and conversion of β^-K^- strains (column B) and β^+K^- strains (column C) always results in β^-K^+ strains. This does not preclude the occurrence of converting phages of the types β^0K^+, β^-K^0 or even β^+K^+ and β^-K^-.

Converting phages in Staphylococcus strains with phage pattern 80/81

In the hope of finding converting phages of these other types, 40 strains of Staphylococcus from our collection and some propagating strains were screened for the β^+K^+ and β^-K^- phenotype. The β^-K^- strains have already been discussed. The strains were taken straight from old stock cultures and spotted on sheep-blood agar and heated-plasma agar. Three strains were presumably β^+K^+, but on plating out they proved to be mixtures of stable variants (called A and B) which were respectively β^-K^+ and β^+K^-, and also differed in phage-typing pattern (Table 5; strains no. 1330, 1451, PS 81). Analysis of these strains showed that the β^-K^+ strains carried a converting phage which was absent from the β^+K^- variant. Both variants also carried a β^0K^0 phage which complicated the demonstration of the converting phages. The phages from strains 1330A and 1451A produced small plaques on strains 1330B and on PS 80. The phage from strain PS 81A was different and produced small plaques on strains 1330B and 57. The phages from the B strains (carrying the β^0K^0 phages) did not react with these indicator strains. The B variants should consequently be placed in column C of row 2 in Table 4 and the A variants in row 3.

When it was realized that the three A strains all had the phage-typing pattern 80/81, 10 other strains which had shown this and related patterns on isolation were taken from our collection, more or less at random. Though some strains were from the same hospital they had been isolated several months apart and can be considered as independent isolations. The strains were taken from old stock cultures as before. In four cases mixtures of A and B variants were found, suggesting that this variation is not rare in old stock cultures. The four strains were nos. 920, 957, 1550, 1629 in Table 5. By chance the same variation was observed in strain PS 80. The variant from strain PS 80 yielded a phage which could be propagated on strains PS 80B or 57. Phage L80 proved to belong to the serological group F in conformity with the results of Rountree (1959).

Several of the B strains from Table 5 could be lysogenized by phage L80 and converted to strains with the properties of A strains. The phages from the A variants thus seem to have the properties of phage L80. Lysogenization of the non-lysogenic strain 57 with phage L80 produced an analogous change in phage pattern. The

Table 5. *Phage pattern, β-toxin and kinase characters of a series of strains of Staphylococcus which were screened as β^+K^+ and proved to consist of a mixture of two variants*

The patterns at the routine test dilution are given. Phage 77 B was derived from phage 77 in 1957 and lyses some untypable strains, notably those of type A (Temple & Blackburn, 1963). w = weak, vw = very weak.

Staphylococcus strain	Date of isolation	Isolated from	Type	Phage pattern	
				In group I	In group III
1456 A	10. v. 1960	Faeces	β^-K^+	81/80	.
1456 B			β^+K^-	81/80	6/7/47/54/83 A/77 B/42 E
1451 A	9. v. 1960	Throat	β^-K^+	52/80/81 (52 A/vw)	.
1451 B			β^+K^-	29/52/80/81/52 A (79/vw)	77 B/83 A
PS 81 A			β^-K^+	80/81	.
PS 81 B			β^+K^-	80/81	77 B/83 A (7/42 D)/42 E/w)
1330 A	6. ii. 1960	Pns	β^-K^+	80 (81/52/w)	.
1330 B			β^+K^-	29/52/52 A/80/81 (79/vw)	77 B/83 A/42 D
1629 A	15. xi. 1960	Nose	β^-K^+	80/81	.
1629 B			β^+K^-	80/81	77 B/83 A/7
957 A	18. viii. 1959	Sputum	β^-K^+	81	.
957 B			β^+K^-	81 (80/52/w)	77 B/7
1550 A	24. ix. 1960	Nose	β^-K^+	80/81/52)52 A/w)	.
1550 B			β^+K^-	80/81/29/52/52 A (79/80/w)	83 A/77 B/42 E/42 D
920 A	21. vii. 1959	Pus	β^-K^+	80/81 (52/vw)	.
920 B			β^+K^-	80/81 (82/52/52 A/vw)	83 A/77 B/7/42 D
PS 80 A			β^-K^+	80/81	.
PS 80 B			β^+K^-	80/81	6/7/77 B/83 A (54/75/w) (42 E/47/vw)
1451 B (L 80)			β^-K^+	80/81/52/52 A	.
1330 B (L 80)			β^-K^+	80/81/52 (52 A/w)	.
57			β^+K^-	29/44/52/52 A/79/80/81	6/7/47/54/75/77/77 B/ 83 A/42 E/42 D (70/w) (73/vw)
57 (L 80)			β^-K^+	52/80/81 (52 A/w)	.
57 (W 46)			β^-K^+	29/44/80/81 (52/52 A/79/w)	6/7/47/(54/75/83 A/w) (70/42 E/53/vw)

relation between conversion of phage pattern and conversion to β^-K^+ is however more or less fortuitous, as is shown by the conversion of strain 57 with phage W46 to β^-K^+ without change of pattern.

Mechanism of conversion

Activity of the converting loci in the prophage state. Phage conversion can be of two types. In the conversion of *Corynebacterium diphtheriae* to toxigenicity the toxin is produced only after phage production and lysis (Barksdale, Garmise & Rivera, 1961; Zabriskie, 1964). The converting gene of the phage seems to be active

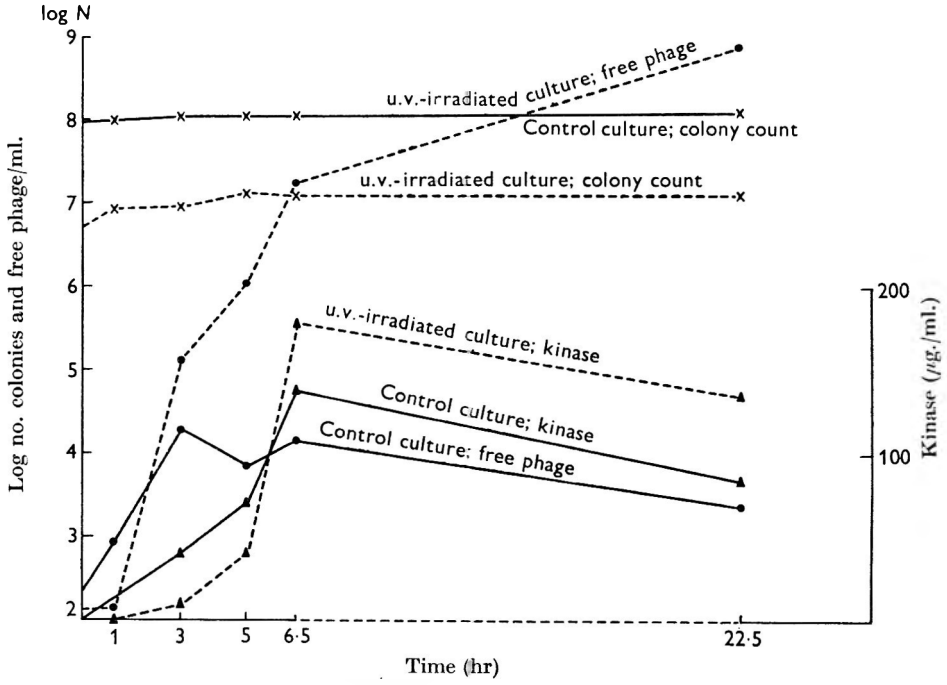

Fig. 1. Staphylococci of strain 269 (269) harvested from a 16 hr culture at 30° were suspended in 60 ml. 0·15 M-phosphate buffer (pH 6·8). Twelve 2 ml. samples were u.v.-irradiated on a rotating disk during 10 sec. at 13 cm. distance from a Philips TUV tube. The u.v-irradiated suspensions were mixed, and 6 ml. of a Todd-Hewitt broth of 5 times usual strength were added. A control suspension was prepared in the same way but without irradiation. Both cultures were incubated for 6·5 hr at 37° with gentle shaking and then allowed to stand at room temperature for a further 16 hr. Colony counts were done by the technique of Miles & Misra. Free phage was counted on a streptomycin-resistant indicator strain of staphylococcus on Difco nutrient agar plates containing 10 μg-streptomycin/ml. Kinase was determined on diffusion plates against a crude standard as described. ●—●, ●- - -●, free phage; × — ×, × - - - ×, colony count; ▲—▲, ▲- - -▲, kinase activity.

during the vegetative phase of phage production but not in the prophage state (see however Pappenheimer, Miller & Yoneda, 1962). On the other hand in the lysogenic conversion of Salmonella which results in changes of antigen the converting gene is active in the prophage state as well as in the vegetative state. In our case the locus converting to loss of β-toxin was obviously active in the prophage state, since β-toxin is not produced by these growing organisms.

To test whether the conversion to kinase production belonged to one or the other group the relation between phage production and kinase production was studied for a lysogenic Staphylococcus strain. Part of a suspension of strain 269 (269) β^-K^+ was induced by u.v.-irradiation; the other part was kept as a control. The staphylococcal colony count, the number of free phage particles and the kinase production were determined in both suspensions during the subsequent period. The results are shown in Fig. 1. The colony count decreased sharply during u.v.-irradiation but remained stationary thereafter. In the induced culture the number of free phage particles—after a latent period of about 1 hr—increased to over 10^7 per ml. In the control culture the number of free phage particles remained about 10^4. Kinase production was about equal in both suspensions. The decrease of kinase activity during the second period was probably due to inactivation by the non-multiplying cocci. As there is no important increase in kinase production in the induced culture, notwithstanding the decrease of the colony count by 90 %, this experiment indicated that kinase was not produced by lysing cocci. Kinase production thus seems to be a property of the lysogenized coccus and the kinase locus on the phage seems to be active in the prophage state.

The number of loci on the phage. The phage locus responsible for conversion to loss of Staphylococcus β-toxin production obviously must be some kind of regulator gene causing suppression of β-toxin production. The kinase locus might be a structural locus or a gene with a de-repressing effect. The correlation between loss of β-toxin production and kinase production might be fortuitous, implying two independent loci on the converting phages, or it might be due to one regulator gene on the phage with a repressive effect on β-toxin production and de-repressive for kinase. Since all isolated phages were β^-K^+ an attempt was made to obtain phage mutants of a different type. U.v.-irradiation of phage 269 for ≤5 sec. resulted in a 25-fold decrease of the number of plaque-forming units. At this time the number of host-range mutants for Staphylococcus strain PS3A was maximal. The u.v.-irradiated suspension was plated on Staphylococcus strain 269. Individual plaques (256) were taken out of the agar layer with a pipette and transferred to 1 ml. of a 2-hr culture of sensitive strain 269. Lysogenization was virtually complete at 24 hr. The cocci were plated on heated rabbit plasma agar for kinase production and on blood agar for β-toxin production. One β^+K^+ strain was found which easily lost phage and reverted to β^+K^-. This strain yielded a phage which converted to kinase production but did not affect the β-toxin production (β^0K^+); that is, it had lost the locus for β-toxin repression. Though only one mutant phage was obtained, this experiment seems to prove that phage 269 contains two independent loci.

DISCUSSION

Fifteen phages separable into at least four unrelated groups have been described here which are able to convert Staphylococcus strains producing β-toxin to the loss of β-toxin production. The converting phages all belong to the serological group F, but at least three F phages are known which do not convert. The loss of ability to produce β-toxin is virtually complete, since cultures grown under optimal conditions for β-toxin production yielded filtrates in which no β-toxin was detected by titration with and without antitoxin (Table 2). The loss of ability to produce

β-toxin is generally accompanied by the gain of staphylokinase (Table 1). Most F phages thus produce the conversion from β^+K^- to β^-K^+, but this conversion has never been observed with phages of other serological groups. Staphylococcal kinase and β-toxin seem to be separate entities (Table 3). The various combinations β^+K^-, β^-K^+, β^-K^- and β^+K^+ can occur in strains in which no phage can be detected (Table 4, row 1) so that structural genes for β and K seem to occur on the bacterial genome. Prophages (not of group F) can also be present in these strains without affecting these characters (Table 4, row 2). These non-converting phages presumably do not carry the genes β or K (β^0K^0 phages). The converting phages of group F generally are of the type β^-K^+, converting β^+K^- strains to β^-K^+ (Table 4, column C) or β^-K^- strains to β^-K^+ strains (column B). No wild-type phages with other combinations were found.

From phage 269β^-K^+ a mutant was obtained which had lost the property of converting to loss of β-toxin production but which had kept the K^+ character, indicating that two loci on the phage genome were involved. Both loci were active in the prophage state. The β-locus may be regarded as a regulator gene, since it suppresses β-toxin production as long as the prophage is present in a strain, though β-toxin production is resumed in strains cured from phage. The kinase locus might be a structural or a de-repressing locus. The presence of a structural kinase locus in strains not carrying phage makes the second possibility slightly more attractive.

The isolation of β^-K^+ and β^+K^- variants from old stock cultures of Staphylococcus strains with phage pattern 80/81, and the demonstration that this variation was due to loss of a converting phage (Table 5) and also affects the phage-typing pattern, seems to indicate that many of these strains of Staphylococcus carry a converting phage. The isolation of the converting F phage L80 from strain PS 80 and the conversions obtained with this phage confirm this. There is an apparent discrepancy in the fact that the converting phages from strains 1330A and 1451A can be demonstrated on strain PS 80 which already carries phage L80. The phenomenon was observed repeatedly. Apart from speculations about more virulent phages overcoming the immunity due to phage L80 we have no explanation.

The data in Table 5 seem to indicate that at least some A strains with phage pattern 80/81 might have arisen from the B pattern by lysogenization with such F phages. The hypothesis that all β^-K^+ strains carry a converting phage is difficult to verify. We have tested many β^-K^+ strains for converting phage without finding any. This may have been due to lack of suitable indicator strains, to double lysogenicity with a minority yield of converting phage, or to the presence of defective prophages. Even the demonstration of a structural kinase locus on the genome of a certain converting phage would probably mean only that it had been derived from a bacterial genome at an earlier period, and certainly does not exclude the presence of a kinase locus on the bacterial genome in another strain. We consequently believe that many Staphylococcus strains carry β and K loci unaffected by phage.

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Reduction of Phenolindo-2,6-dichlorophenol in Dark and Light by the Blue-Green Alga, *Anabaena variabilis*

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SUMMARY

The reduction of phenolindo-2,6-dichlorophenol (PICP) by the blue-green alga *Anabaena variabilis* was examined in the dark and in the light. The two reductions differed in order with respect to PICP, in pH optimum, response to cyanide and iodoacetate, and in sensitivity to starvation and lysozyme treatment of the organism. The reduction in the dark was suppressed at light intensities of 300 ft.-candles. These results are discussed in relation to work on the photo-inhibition of respiratory processes in this and other micro-organisms.

INTRODUCTION

The blue-green algae (Cyanophyceae) are structurally the simplest of the algae. Electron microscope studies have shown their resemblance to the bacteria (Ris & Singh, 1961; Wildon & Mercer, 1963) in that they lack morphologically distinct organelles such as nuclei, mitochondria and chloroplasts. The main reason for their inclusion among the algae is their plant-like, rather than bacterial, type of photosynthesis; they possess chlorophyll *a* rather than bacteriochlorophyll and use water as the ultimate reductant. The Hill reaction, the photo-stimulation of O_2 production on the addition of artificial oxidants, occurs not only with chloroplasts but also with intact green algae (Clendenning & Ehrmantraut, 1950) and blue-green algae (Thomas & De Rover, 1955). While studying the physiology of intact *Anabaena variabilis* we examined spectrophotometrically the reduction of a typical Hill oxidant, phenolindo-2,6-dichlorophenol by this alga, in the light and in the dark, and have attempted to characterize the two types of reaction. Recent workers (Fredricks & Jagendorf, 1964; Krogmann & Susor, 1964) studied Hill reactions catalysed by chlorophyll-containing fragments of blue-green algae but with particular reference to the role of phycocyanin. Fredricks & Jagendorf (1964) noted a dark reduction of 2,3,6-trichlorophenolindophenol by chlorophyll-containing fragments; however, they suggested that this was probably due to the presence of endogenous reducing compounds. A preliminary account of some of the experiments reported has been published (Carr & Hallaway, 1964).

METHODS

Organisms. Anabaena variabilis (Kützing) was kindly supplied by Professor J. Myers (Department of Botany and Zoology, University of Texas, U.S.A.) and *Chlorogloea fritschii* (Mitra) and *Chlorella pyrenoidosa* by Professor G. E. Fogg

(Department of Botany, Westfield College, London), *Nostoc muscorum* (1453/12) and *Anabaena cylindrica* (1403/2) were obtained from the Culture Collection of Algae and Protozoa, Department of Botany, University of Cambridge.

Chemicals. Phenolindo-2,6-dichlorophenol (PICP) and lysozyme hydrochloride were bought from British Drug Houses Ltd. (Poole, Dorset) and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) from K. and K. Laboratories, Inc., New York.

Medium and growth conditions. All organisms were grown on Medium C (Kratz & Myers, 1954) supplemented with 0·05 % (w/v) $NaHCO_3$. This provided an essentially inorganic medium, a small amount of sodium citrate (0·165 g./l.) being the only organic constituent of medium C; carbon dioxide was the source of carbon for growth. Stock cultures were maintained on agar (1 %) slopes of this medium and cultures for experimental purposes were grown at 32–34° in 500 ml. medium in 2 l. penicillin pots, illuminated by 60 W. daylight strip lights at a distance of 9 in., gassed with a mixture of air + CO_2 (95 + 5, by vol.) and gently shaken. Some earlier cultures were gassed with a mixture of N_2 + CO_2 (95 + 5, by vol.). The behaviour of algae with respect to PICP reduction when grown in either gas mixture was the same. Under the conditions described *Anabaena variabilis* grew to a population density of equiv. 0·5–1·0 mg. dry wt./ml. in 4–6 days. Algae were harvested at room temperature by centrifugation at 500*g* for 4 min. washed in half their initial volume of water, recentrifuged and suspended in water to equiv. 20 mg. dry wt./ml. Growth was measured turbidimetrically, and a calibration curve used to relate EEL colorimeter reading to mg. dry wt./ml.

Measurement of reduction of oxidants. The reduction of oxidants in the light and in the dark was measured spectrophotometrically in a Unicam S.P. 700 recording spectrophotometer. A glass cuvette, fitted with a ground glass stopper, containing equiv. 1·5–3·0 mg. dry wt. algae, oxidant to give an initial extinction of 0·8–1·4 (e.g. 0·005 % PICP), KH_2PO_4 + K_2HPO_4 buffer (pH 6·0) final concentration of 0·025 M, in a total volume of 2·75 ml. was illuminated with frequent agitation at a distance of 15 cm. from a 100 W. tungsten bulb yielding a light intensity of about 300 ft.-c. at the cuvette and a temperature of 27–30°. The rate of reduction of oxidant was measured at the absorption maximum of its oxidized form (606 mμ at pH 6·0 for PICP) against a blank containing algae + buffer only. The rate of reduction in the dark was measured under the same conditions but with exclusion of light; the time for the measurement of extinction in the spectrophotometer did not exceed 20 sec. Since reduced PICP is oxidized by air, in some experiments the reactions were done under nitrogen by using a modified Thunberg top to the cuvette.

Production of light of (*a*) *known spectral region and* (*b*) *known intensity.* (*a*) When light of a particular spectral region was required the experimental cuvette was shielded from laboratory light and illuminated by using appropriate filters (see Results). Data on the transmission characteristics for the filters were obtained from *Kodak Wratten Filters*, 2nd edn, 1961, and from Chance-Pilkington Optical Works, St Asaph, Flint.

(*b*) Illumination of different intensities was obtained by using lamps of different wattage (25–300) or by interposing one or more wire-gauze screens between a 100 W bulb and the cuvette. In each case the intensity of incident light on the cuvette was measured by a Multi-Lux exposure meter.

Absorption spectra of pigments of Anabaena variabilis. Photosynthetic pigments

were separated into a (chlorophyll + carotenoid-bearing) sedimentable fraction and a soluble phycocyanin-containing fraction. The algae were sonically disintegrated in a M.S.E. 60 W. disintegrator for 3 min. and then centrifuged at 50,000*g* for 90 min. The phycocyanin was obtained in the clear blue supernatant fluid and chlorophyll and carotenoids were sedimented in the pellet. The latter pigments were extracted from the pellet by a mixture of acetone + methanol (7 + 2, by vol.) and their spectra recorded by a Unicam SP. 800.

RESULTS

The reduction of oxidants by microalgae

The reduction of various oxidants by different microalgae was examined under the standard conditions. Phenolindo-2,6-dichlorophenol (PICP) was reduced in the light and dark by all the algae investigated (*Anabaena variabilis*, *A. cylindrica*, *Nostoc muscorum*, *Chlorogloea fritschii* and *Chlorella pyrenoidosa*); in each case light

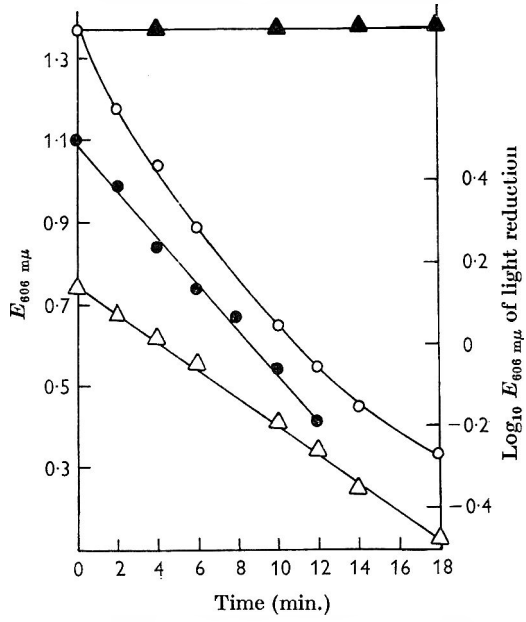

Fig. 1. Reaction course of PICP reduction by *Anabaena variabilis* in light and dark. Reactions measured under the standard conditions with equiv. 1·8 mg. dry wt. algae per cuvette. ○, Reaction in light pH 6·0; △, $\log_{10}$ extinction of light reaction; ●, reaction in dark pH 6·5; ▲, reaction in dark and light in presence of growth media, after removal of algae. k for light reaction = $7{\cdot}4 \times 10^{-4}$ sec^{-1}/mg. dry wt. algae.

appeared either to accelerate a light-independent reduction, or to initiate a kinetically different reaction. Methylene blue was reduced, in the presence and absence of light, by *N. muscorum* and *A. variabilis* but not by *A. cylindrica*. Ferricyanide was not reduced by *A. variabilis*, either at pH 6 or 7, $\pm 0{\cdot}35$ mM-$MgCl_2$ nor by Chlorella (Clendenning & Ehrmantraut, 1950), although it is utilized as a Hill oxidant by isolated chloroplasts. Although benzoquinone was reduced by *A. variabilis* it is unsuitable for use with intact algae since it is known to damage them rapidly and irreversibly (Clendenning & Ehrmantraut, 1950). The reduction of PICP by *A. variabilis* was selected for further examination.

Kinetics of the reduction of PICP in dark and light by Anabaena variabilis

The rate of PICP reduction by *Anabaena variabilis* was linear with time in dark but decreased apparently exponentially in light; the corresponding logarithmic plot for the reduction in light was linear (Fig. 1). This suggested that the reduction in light was of first order and that in dark was of zero order with respect to PICP concentration. This was confirmed by the results obtained when the PICP concentration was varied in light and dark. A threefold increase in PICP concentration did not alter the rate of reduction in dark but increased proportionately the rate in light (Fig. 2*a*). In dark and in light the rate of reduction was directly proportional to the

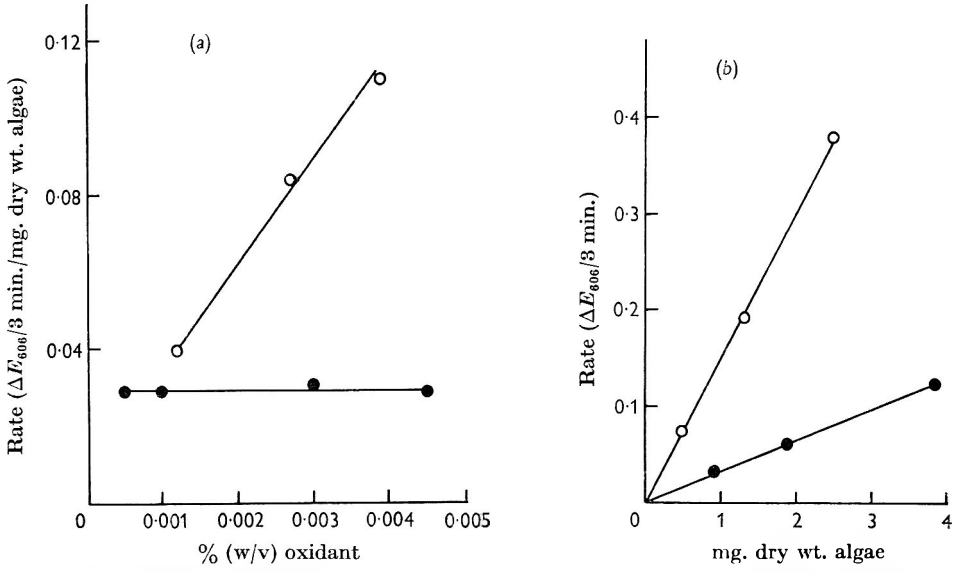

Fig. 2. Effect of (*a*) PICP concentration, (*b*) algal concentration on rate of PICP reduction. ○, Reduction in the light; ●, reduction in the dark. Measured under the standard conditions except for the variable studied.

concentration of organism (Fig. 2*b*). The question arises, does the dark reduction continue in light or is it wholly or partially suppressed? The available evidence supports the hypothesis that at intensities of 300 ft.-c. and greater, light completely suppressed the dark reduction. Under the standard light conditions the rate of reduction decreased exponentially with time. When the decrease in extinction was corrected for a postulated simultaneous light-independent reduction the corrected changes fitted no standard equation of reaction kinetics. Furthermore, when the organisms were allowed to reduce part of the PICP in light and were then transferred to dark, only a slow rather erratic reduction took place (Fig. 3). In contrast, transferring the system to light after a period of reduction in dark merely induced the expected sharp change in the kinetics of the reaction (Fig. 3); the rate constant for light reaction was unaffected by the preliminary reduction in dark.

However, suppression of the dark reduction by light depended on the light intensity and the ratio of algae to PICP in the cuvettes. At a light intensity of 20 ft.-c. the reaction was indistinguishable from that in the dark; at 70 ft.-c. the light

reaction was first order but a subsequent dark reduction was only partially suppressed. The mechanism of the suppression by light is not known; illumination of the organisms, the PICP, or both, separately, before transferring to the dark and mixing, had no inhibitory effect on the reduction which then took place in the absence of light.

None of the reducing activity of *Anabaena variabilis* is released into the medium, since neither the growth medium from which the organisms had been harvested, nor water in which they had been suspended had any detectable effect on PICP in dark or light (Fig. 1.).

Effect of pH value on PICP reduction

The form and optimum of the effect of pH value on the rate of reduction in light and in dark differed (Fig. 4); the pH optimum of the dark reduction was 6·5, that of the light 6·0.

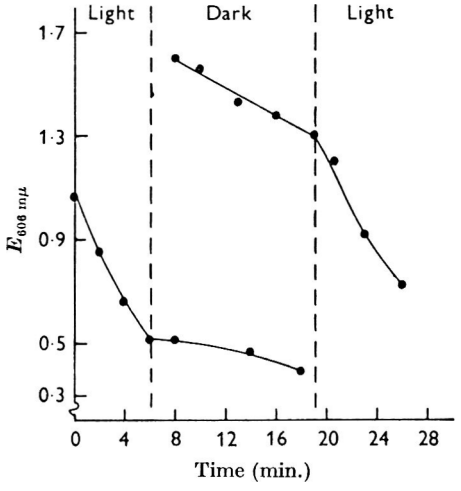

Fig. 3

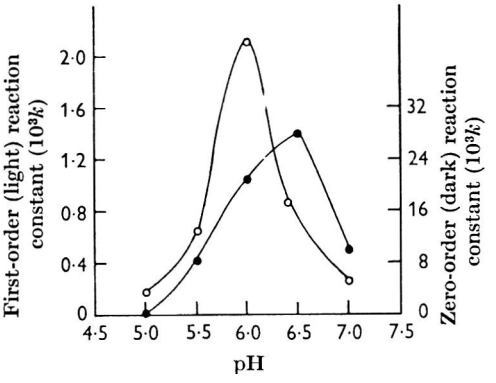

Fig. 4

Fig. 3. Alternate effects of light and dark on PICP reduction by *Anabaena variabilis*. Reactions carried out under the standard conditions.

Fig. 4. Effect on pH value on light and dark reduction of PICP by *Anabaena variabilis*. ○, Reduction in light; ●, reduction in dark. Reactions measured under the standard conditions except for the differences in pH value.

Effect of light intensity and temperature

The rate of the light reaction at two temperature ranges and at different intensities of light is shown in Fig. 5. Under the standard conditions (temperature 27–30°) the rate of reduction increased with increasing light intensity over the range 45–300 ft.-c. Increasing the temperature to 35–36° increased the reduction rate at these light intensities, which suggests that light was not the sole limiting factor (Fig. 5). However, at the higher temperature, increasing the light intensity from 150 ft.-c. to 300 ft.-c. had relatively little affect on the reduction rate, so that under these conditions the light intensity was no longer rate limiting.

Utilization of light of different spectral regions

By using different light sources in association with appropriate filters *Anabaena variabilis* was exposed to light of various wavelengths; the regions selected were those of maximum absorption of the *A. variabilis* pigments (Fig. 6). It was not possible with the apparatus available to adjust the light source so that the algae were illuminated with light of different wavelengths but of equal intensity, hence quantitative comparison is impossible. However PICP reduction was increased, relative to reduction in the dark, by light of each spectral region used. This suggests that light absorbed by all three pigment types was effective in promoting PICP reduction.

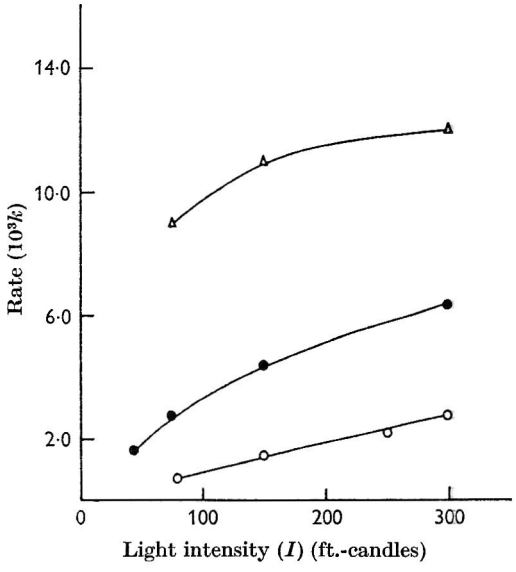

Fig. 5

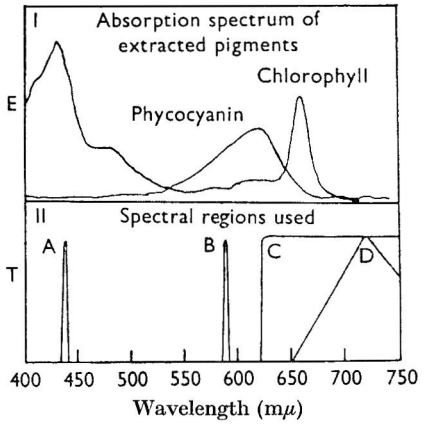

Fig. 6

Fig. 5. Effect of light intensity, at two temperatures on PICP reduction by *Anabaena variabilis*. The reactions were done under the standard conditions except that light intensity (*I*) was varied by two means. ○, *I* varied by using lamps of different wattage; temperature 27–30°; ●, *I* varied by using a 100 W lamp and a series of wire-gauze screens; temperature 27–30°; △, *I* varied with wire-gauze screen; temperature 35–36°.

Fig. 6. (I) Absorption spectra of pigments of *Anabaena variabilis* and (II) spectral regions used in PICP reduction. (I) shows the absorption spectrum of the pigments extracted from *Anabaena variabilis*. The absorption below 500 mμ is attributable to carotenoids and chlorophyll a, the peak near 600 mμ to phycocyanin and the peak in the 660 mμ region to chlorophyll *a*. (II) is a diagrammatic representation of the transmission characteristics of four combinations of filters and light sources devised to provide light of spectral regions absorbed by *Anabaena* pigments.

Spectral region	Light source	Filter
A	Mercury lamp	Kodak Wratten 98
B	Sodium lamp	None
C	Mercury lamp	Kodak Wratten 29
D	Tungsten lamp	Kodak Wratten 29, Chance OX 7

Effect of inhibitors

The difference between light and dark reaction kinetics, in pH optimum, and the existence of a light inhibition of the dark reduction, all indicate that the reduction reactions in light and dark differed in mechanism and presumably also in the source of hydrogen used in the reaction. This possibility was examined by testing the effect of various compounds on the light and dark reductions. The reactions differed in their response to cyanide (0·5–1·0 mM) and iodoacetate (0·7–1·4 mM) both of which stimulated the dark reduction but did not affect the light reduction. Since in the dark PICP is probably acting as an alternative electron acceptor to oxygen stimulation by cyanide, which inhibits cytochrome oxidase is not unexpected; stimulation by iodoacetate is not easy to explain since if it were inhibiting glycolysis this would retard the flow of electrons to PICP. Neither 0·7 mM-hydroxylamine hydrochloride nor 0·1 mM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) inhibited the reduction in light; both are inhibitors of the oxygen producing stages of photosynthesis but DCMU does not inhibit photoreductions (Gingras, Lemasson & Fork, 1964; Hoch, Owens & Kok, 1963).

Reduction of PICP by starved Anabaena variabilis

The oxygen consumption and PICP reduction by organisms that had been starved of CO_2 by gassing with CO_2-free air for 5 hr before harvesting were examined and compared with organisms from the same culture which had been gassed with 5% (v/v) CO_2 + 95%(v/v) air until harvested. Starvation did not alter the rate of the light reaction significantly, but the dark reaction was no longer linear and ceased after about half the PICP had been reduced. The endogenous respiration of the starved algae was less than 50% of that obtained with normal organisms. These observations accord with the suggestion that the endogenous reserves are the source of the electrons for the dark reduction, but not for the reaction in light.

Table 1. *Effect of lysozyme treatment of Anabaena variabilis on PICP reduction*

A suspension of algae equiv. 20 mg. dry wt./ml. was incubated with various concentrations of lysozyme for 30 min. at 34°; after this the algae were rapidly sedimented by centrifugation, washed in 3 ml. water and recentrifuged, finally being resuspended in their initial volume (1 ml.) of water. The rate of reduction of PICP was measured under standard conditions.

Concentration of lysozyme (mg./ml.)	Activity of PICP reduction remaining after incubation (%)	
	Dark	Light
0	100	100
0·3	56	100
1·0	32	49
2·0	12	39

Reduction of PICP by Anabaena variabilis treated with lysozyme

Incubation of a washed suspension of algae with lysozyme resulted in release of phycocyanin and a rapid decrease in the rate of both dark and light reduction of PICP. The reduction in dark appeared to be the more sensitive to lysozyme treat-

ment. The decrease in rate of the reaction in dark and light after 30 min. incubation with different concentrations of lysozyme is shown in Table 1. Addition of phycocyanin to inactivated preparations did not restore the ability to reduce PICP.

If the PICP was acting as final electron acceptor additional to oxygen it might be expected that lysozyme treatment which retarded the transfer of electrons to PICP would also retard the respiratory transfer of electrons to oxygen. However, oxygen uptake due to endogenous respiration of *Anabaena variabilis* was not decreased by incubation with lysozyme, even when 75% of the phycocyanin had been lost and PICP reduction in light and dark was barely detectable (Fig. 7). The initial loss of

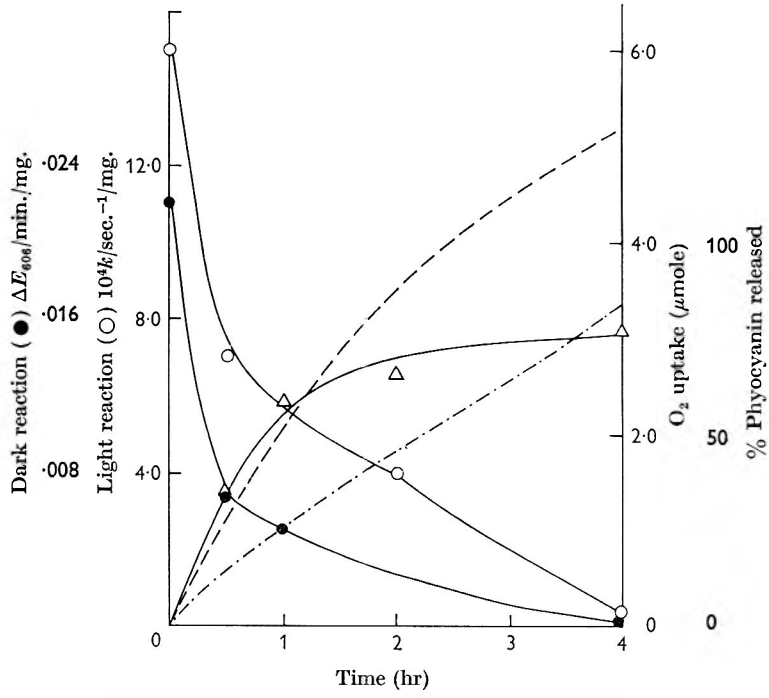

Fig. 7. Effect of lysozyme treatment on release of phycocyanin, respiration and light and dark reduction of PICP by *Anabaena variabilis*. ○, PICP reduction in light; ●, PICP reduction in dark; - - - -, endogenous respiration in the presence of lysozyme; ·····, endogenous respiration without lysozyme treatment; △, % phycocyanin released. The PICP reductions were measured on lysozyme-treated algae under the standard conditions. Endogenous respiration was measured by O_2 uptake by the direct manometric method. The release of phycocyanin into the supernatant fluid by lysozyme treatment was estimated by measuring the extinction at 610 mμ (E_{610}) and comparison with the extinction of the total phycocyanin obtained by sonic disruption of the algae followed by centrifugation at 50,000 *g* for 90 min.

phycocyanin from the algae into the medium was rapid; over 50% of the total phycocyanin was released during the first hour of incubation, coinciding with the marked decrease in ability to reduce PICP. There was no indication that the lysozyme treatment had lysed the organisms, since they could still be sedimented by centrifuging at 500*g* for 5 min., and appeared intact by microscopic examination.

DISCUSSION

Phenolindo-2,6-dichlorophenol (PICP) has often been used as an artificial oxidant in studying both respiratory and photosynthetic electron transfer. With an E_0' of +295 mV. at pH 6·0 it would be reduced by flavoproteins and could be oxidized by some cytochromes of the respiratory chain. The point at which PICP may be reduced in photosynthetic electron transport is still not clear (Gromet-Elhanan & Avron, 1964). Our results show that *Anabaena variabilis* can reduce PICP in the dark and light, that the two reductions differ, and that light suppresses the dark reaction. It seems probable that the ultimate reductant in the light is water, and in the dark is the endogenous substrates of the organism, but the mechanisms of the two reductions and of the light suppression of the dark reaction are not yet established.

Suppression of respiratory oxygen uptake by light has been noted for both blue-green algae (Brown & Webster, 1953, Hoch, Owens & Kok, 1963) and purple bacteria (van Niel, 1941; Fork & Goedheer, 1964). To explain the phenomenon Brown & Webster (1953) suggested that either the photosynthetically produced oxygen was utilized within the organism (an explanation which cannot hold for the bacteria in which oxygen is not a product of photosynthesis), or that light inhibited respiratory electron transfer. Assuming that PICP is acting in both presence and absence of light merely as an electron acceptor, then our results support the latter suggestion.

The interaction of the light and dark reduction of PICP by *Anabaena variabilis* could be another example of the structural and functional linkage of photosynthesis and respiration found in procaryotic organisms and discussed by Stanier & van Niel (1962). Procaryotic organisms, bacteria and blue-green algae, differ from eucaryotic organisms in that their genetic material is not enclosed by a defined membrane separating it from the cytoplasm nor do they possess membrane bound organelles specialized for different functions, e.g. chloroplasts for photosynthesis or mitochondria for respiration. Hence the electron transport chains of procaryotic organisms may serve both in respiration and photosynthesis so that photosynthetic electron transport may exclude respiratory electron transfer (see Horio & Kamen, 1962).

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The Autolysis of *Clostridium sporogenes*

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SUMMARY

Washed *Clostridium sporogenes* lysed during storage at 2° or on incubation at 37° in various buffered salt solutions. The lysis was accompanied by a marked increase in adenosine triphosphatase activity (ATPase), due to an enzyme bound to the cytoplasmic membrane. The lysis in buffered salt solutions was most rapid in young vegetative organisms and negligible in organisms from cultures incubated for 30 hr. No lysis occurred when organisms were incubated in buffered sucrose, but young organisms formed spheroplasts under these conditions. Both young and older organisms incubated in sucrose lysed when diluted in water. After lysis the residue of older organisms appeared structurally similar to cell-wall membranes prepared by crushing the organisms. Organisms incubated in EDTA or salt solutions containing sulphydryl compounds did not lyse during the incubation or on subsequent dilution with water. It is suggested that lysis was mainly due to changes in the composition or structure of the cell wall and not to alterations in the protoplasmic membrane.

INTRODUCTION

The lysis of bacteria can occur without the addition of a lytic agent either in the growth medium or after separation from it. Such autolysis may be preceded by the formation of rounded osmotically sensitive forms (spheroplasts or protoplasts; McQuillen, 1960), but often the organisms may lyse without spheroplast formation (Collins, 1964). In either case, the autolysis is associated with changes in the cell wall leading to loss of rigidity when spheroplasts are formed or a change in association of the cell wall and the permeability barrier when they are not formed (Collins, 1964). The present work is concerned with factors which control the autolysis of *Clostridium sporogenes* which occurred after collecting, washing and storing the organisms in various buffered media. Lysis was followed by measuring the release of cell components and estimating the activity of a membrane-bound ATPase which is apparently inactive in intact organisms owing to impermeability to ATP.

METHODS

Growth and handling of organisms. The organism used was a strain of *Clostridium sporogenes* isolated from mouse intestinal microflora and characterized by its ability to utilize cholesterol (Galli & Carini, 1962). It was maintained and grown on

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the following medium: Oxoid tryptone, 20 g; Oxoid yeast extract, 6 g; glucose, 20 g.; sodium acetate, 10 g.; KH_2PO_4, 4·5 g.; ascorbic acid, 1 g.; salt B solution (Barton-Wright, 1952), 5 ml. The components were dissolved in 1 l. water and adjusted to pH 7·6 with NaOH. Usually 10 l. batches were grown in 10 l. narrow-necked Pyrex bottles filled to the top with the medium and autoclaved for 10 min. at 115°. After incubation at 37° the organisms were collected by centrifugation and washed once in a solution containing 0·9% (w/v) NaCl and 0·2M-tris + maleate buffer (pH 7·0). The organisms were resuspended, packed by centrifugation for 30 min. at 8000*g* and then suspended smoothly in a suitable medium by the aid of a Konte's type glass homogenizer (Konte's Glass Works, Vineland, New Jersey, U.S.A.) to contain the equivalent of 0·1 g. dry wt. organisms/ml.

Estimations of lysis. Suitable volumes of the above suspension of organisms were placed in the medium to be studied, in glass-stoppered tubes. To follow lysis, samples were taken at intervals and their extinction measured at 540 mμ (E_{540}) in a Unicam SP600 spectrophotometer. The extinction was expressed as

$$\frac{E_{540}\text{ initial} - E_{540}\text{ final}}{E_{540}\text{ initial}} \times 100$$

and is referred to as '% lysis'. Samples were prepared at the same time for light microscopy and examined immediately by phase contrast or fixed by heat and stained by Gram's method. Samples for electron microscopy were fixed in Palade buffered osmium solution (Palade, 1952), washed twice with water and mounted on Formvar covered grids. The grids were examined at magnifications of ×600–17 000 in the Akashi Tronoscope. When the release of components from organisms to the suspending fluid was to be measured the samples were centrifuged at 2° for 15–20 min, at 18,000*g* and the component estimated in the clear supernatant fluid.

Chemical estimations. Protein was estimated with the Folin–Ciocalteau reagent (Lowry, Rosebrough, Farr & Randall, 1951); total carbohydrate by the method of Dische & Shettles (1948); deoxyribonucleic acid by the method of Burton (1956) and ribonucleic acid by the method of Mejbaum (1939). Lipid was extracted from preparations, which had been dried from the frozen state, with methanol + chloroform (2 + 1 by vol.), the extract washed with water, dried over Na_2SO_4, evaporated to dryness under a stream of nitrogen, dried *in vacuo* over paraffin wax, and the residue weighed. The lipid residue was redissolved in chloroform and the total P estimated for after ashing in a perchloric + sulphuric acid mixture (2 + 1 by vol.) by the method of Fiske & SubbaRow (1925). All constituents are expressed as mg./mg. dry wt. organisms estimated after drying a sample at 110° to constant weight, and subtracting the dried weight of the suspending medium.

Estimation of enzyme activity. The activity of ATPase was estimated by assuming that ATP is hydrolysed according to the reaction $ATP + H_2O \rightarrow ADP + H_3PO_4$. Usually 0·1 ml. of the cell preparation was incubated at 37° in a mixture containing ATP 1·0 μmole, 0·5 ml. 0·2M-tris + maleate buffer (pH 7·0), $MgCl_2$ 1·0 mole; in total vol. 1·0 ml. After incubation the tubes were placed in ice, cooled to 2°, and 1·0 ml. cold 5% (w/v) perchloric acid added. The tubes were centrifuged and phosphate estimated in 0·5 ml. of the supernatant fluid by the method of Fiske & SubbaRow (1925).

RESULTS

Lysis of stored suspensions of organisms

Suspensions of organisms which had been incubated for 16 to 24 hr were stored in 0·2 M-tris + maleate buffer (pH 7·0) containing 0·9 % (w/v) NaCl at 2° for periods up to 7 days, during which time they became very viscous. The viscosity was rapidly decreased by the addition of crystalline DNase (1 mg./100 ml. suspension), and was presumably due to the release of DNA from the organisms. After treatment with DNase the suspension was centrifuged for 30 min. at 28,000*g*. The supernatant fluid contained the bulk of the cell protein DNA and RNA. The solid residue contained high ATPase activity (Table 1) and in the electron microscope was similar in appearance to cell-wall membrane preparations obtained by crushing fresh organisms in the Hughes press and treatment with DNase (Hunt, Rodgers & Hughes, 1959). In the light microscope by phase contrast the organisms appeared to be little changed, but when stained by Gram's method most organisms were Gram-negative, instead of Gram-positive as at the beginning of the storage period. There was no apparent formation of spheroplasts at any time in such suspensions. A similar but more rapid lysis was observed when the suspensions were incubated at 37°: complete lysis occurred in 16–18 hr. Most of the subsequent experiments were done at 37°.

Table 1. *Lysis of Clostridium sporogenes vegetative forms and corresponding ATPase activity*

Organisms were stored for 7 days at 2° in a 0·9 % NaCl + 0·2 M-tris + maleate buffer (pH 7·0). Treated with DNase, centrifuged and ATPase estimated in the usual manner. The ATPase activity is compared with that of the fresh organisms after crushing in the Hughes press.

	ATPase activity (μmole Pi/mg. dry wt./hr.)	Protein in supernatant fluid (mg./mg. dry wt. organisms)
Whole fresh organisms	0·10	0·04
Outer shell (by autolysis)	0·30	0·20
Outer shell (by Hughes press)	0·45	0·40

The effect of sucrose on lysis

Suspensions of organisms in concentrations of sucrose from 0·5 to 1·5 M were incubated at 37° for 18 hr. Slight lysis was observed in the lowest concentration but no lysis occurred in 1·0 M-sucrose as judged by extinction and protein determinations. However, when such suspensions were diluted with water to decrease the concentration of sucrose below 0·2 M, most of the remaining organisms lysed almost immediately. The ATPase of suspensions was negligible before dilution but increased rapidly upon dilution and was equal to that of organisms incubated in the absence of sucrose (Table 2). In the light microscope and in the electron microscope organisms incubated in sucrose appeared to be unchanged, but were found to be empty upon dilution in water (Fig. 1). NaCl at various concentrations had no effect on lysis. Na ethylenediaminetetra-acetate (EDTA, 500 μg./ml.) inhibited lysis completely. Organisms incubated with EDTA did not lyse on dilution in water nor did their

ATPase activity increase. The addition of $CaCl_2$, $MgCl_2$, $MnCl_2$ (all 0·01 M) as well as EDTA allowed lysis to proceed (Table 3).

The effect of sylphydryl compounds on lysis

The addition of cysteine, mercapto-acetate, aminoethylthiouronium chloride (AET) or mercapto-ethanol to the incubation mixture in concentrations from 0·05 to 0·004 M slowed or prevented the lysis (Table 4). In the optimum mercapto-acetate concentration (0·02 M) organisms appeared to be unchanged microscopically after incubation at 37° for 18–20 hr and even after dilution in water the organisms appeared normal. There was no increase in ATPase activity in organisms incubated with the sulphydryl compounds and then diluted in water; surprisingly, mercapto-ethanol did not affect lysis. There was no marked difference between organisms shaken and incubated in wide flasks exposed to the air or in narrow sealed tubes. Likewise collecting and washing the organisms in media containing SH-compounds

Table 2. *The effect of sucrose concentration on lysis of stored organisms*

Washed suspensions of *Clostridium sporogenes* vegetative forms were suspended in 0·2 M-tris + maleate buffer pH 7·0 with or without sucrose as indicated. After incubation at 37° for 18 hr the organisms were separated by centrifugation and the ATPase activity of a portion estimated as usual (before dilution), and the protein content of the supernatant was also estimated. Another portion of the organisms was suspended in water, centrifuged, and the ATPase activity of the solid and the protein content of the supernatant estimated (after dilution). Control was not incubated.

Sucrose concn. (molar)	ATPase activity (μmole Pi/mg. dry wt. organisms/hr)		Protein in supernatant (mg./mg. dry wt. organisms)	
	Before dilution	After dilution	Before dilution	After dilution
Control	0·20–0·10	—	0·04–0·08	—
None	0·50–0·35	0·58	0·19–0·81	—
0·5	0·22	0·62	0·10	0·25
0·75	0·13	0·43	0·19	0·30
1·0	0·05	0·30	0·11	0·20
1·5	0·17	0·54	0·10	0·20

Table 3. *The effect of EDTA and metal ions on lysis of organisms*

Washed suspensions of *Clostridium sporogenes* were suspended in 0·2 M-tris + maleate containing 0·9 % (w/v) NaCl and the addition shown below. After 18 hr at 38° the suspension was centrifuged and suspended in buffer. ATPase activity was estimated in a portion (before dilution) and another portion was diluted in 10 vol. water, centrifuged and ATPase estimated in the solid residue (after dilution). Percentage lysis was estimated as described in the text.

Addition	Lysis (%)	ATPase activity (μmole Pi/mg. dry wt. organisms/hr)	
		Before dilution	After dilution
EDTA (500 μg./ml.)	0	0·17	0·26
EDTA (500 μg./ml.) and 0·01 M-$MgCl_2$ or -$MnCl_2$ or -$CaCl_2$	33	0·55	0·64

or rigid exclusion of oxygen by nitrogen during collection, washing and incubation, made no difference to the rate of lysis. The protective effect of SH-compound therefore did not appear to be simply due to effects of oxygen on this strict anaerobe.

The effect of age of culture on lysis

Cultures of *Clostridium sporogenes* were harvested at different times corresponding to the late lag (4 hr), exponential growth (8–12 hr) and stationary (12–30 hr) phase of growth, and their lysis followed by extinction and microscopic examinations under the usual conditions when suspended in buffer alone, buffer + sucrose, or buffer + mercapto-acetate. In buffer alone the lysis was greater in the youngest organisms and least in the oldest, in fact organisms harvested at 30 hr did not lyse

Table 4. *The effect of sulphydryl compounds on lysis of organisms*

Clostridium sporogenes vegetative forms suspended in 0·2 M-tris + maleate, pH 7·0 with 0·9 % (w/v) NaCl solution, were incubated at 37° with sulphydryl compounds and their ATPase activity measured as usual before and after dilution in water.

Sulphydryl compounds	Lysis (%)	ATPase activity (μmole Pi/mg. dry wt. organisms/hr) Before dilution	ATPase activity (μmole Pi/mg. dry wt. organisms/hr) After dilution in water
Cysteine (0·02 M)	0	0·15	0·20
Mercapto-ethanol (0·02 M)	60	0·40	0·36
Aminoethylthiouronium chloride (0·2 M)	0	0·13	0·20
Mercapto-acetate (0·02 M)	0	0·17	0·24
Mercapto-acetate (0·05 M)	0	0·04	0·15
Mercapto-acetate (0·004 M)	70	0·56	0·40

Table 5. *The effect of time of harvesting organisms on lysis*

Clostridium sporogenes vegetative forms were harvested from the culture medium at the times indicated, incubated in 0·02 M-tris + maleate buffer, pH 7·0, for 18 hr and lysis followed by measuring the extinction.

Time of harvesting (hr)	Suspending medium: Buffer alone	Suspending medium: Buffer + sucrose (M) Lysis (%)	Suspending medium: Buffer + 0·02 M mercapto-acetate
4	86	20	0
8	92	20	0
12	64	20	0
16	58	18	0
30	15	6	0

appreciably during incubation for 18 hr (Table 5). In buffered sucrose, the youngest organisms (4–8 hr) slowly lysed but this was accompanied by the appearance of some spheroplasts (Fig. 2). The older organisms (18–30 hr) did not show such spheroplast formation. The spheroplasts from young organisms in buffer + sucrose lysed instantly when diluted in water, leaving typical 'ghost' forms (Fig. 3). In the electron microscope most of the spheroplasts from young organisms appeared to be empty even before dilution in water. This was because they lysed upon the addition

of fixative, no satisfactory way was found to preserve them. In contrast, apparently unlysed organisms and older organisms upon dilution formed cell-wall membranes (Fig. 4). Both young and older organisms incubated in buffered mercapto-acetate (0·03 M) remained intact, as judged microscopically, by extinction and protein determinations (Table 6).

Table 6. *The effect of harvest time of organisms on the rate of lysis*

Clostridium sporogenes was harvested at the times indicated and the organisms incubated at 37° in 0·2 M-tris + maleate, pH 7·0 with additions as indicated. Lysis was followed by measuring E_{540}

Time of harvesting (hr)	Addition	Time of incubation: 2 hr	4 hr	8 hr	18 hr
		% lysis			
4	None	92	92	92	92
	Sucrose (M)	10	16	20	40
	Buffer + mercapto-acetate (0·02 M)	0	0	0	0
8	None	76	96	96	96
	Sucrose (M)	6	8	10	25
	Mercapto-acetate (0·02 M)	0	0	0	0
16	None	2	4	4	54
	Sucrose (M)	2	2	2	25
	Mercapto-acetate (0·02 M)	0	0	0	0

Table 7. *Relation of period of incubation of cultures to ATPase activity during lysis*

Clostridium sporogenes was harvested, suspended in 0·2 M-tris + maleate, pH 7·0, with additions as indicated. Their ATPase activity was estimated in the presence of M-sucrose in the incubation mixture after incubation at 37°. Enzyme activities were also estimated after dilution in water.

Age of cells	Addition	Time of culture incubation: 0 hr	4 hr	17 hr	After dilution in water
		ATPase activity (μmole Pi/mg. dry wt./hr)			
4	None	0·20	0·50	0·50	0·60
	Sucrose (M)	0·20	0·24	0·30	1·00
	Mercapto-acetate (0·02 M)	0·20	0·20	0·20	0·28
8	None	0·10	0·60	0·60	0·60
	Sucrose (M)	0·10	0·22	0·40	1·2
	Mercapto-acetate (0·02 M)	0·10	0·10	0·10	0·25
16	None	0·20	0·30	0·50	0·50
	Sucrose (M)	0·20	0·22	0·22	0·62
	Mercapto-acetate (0·02 M)	0·20	0·20	0·20	0·22

In following the ATPase of young cultures it was found that many batches lysed in less than 20 min. in the enzyme assay incubation mixture, this was completely prevented by the addition of sucrose (to 1·0 M). There was no apparent effect of

sucrose on ATPase activity. With sucrose in the incubation mixture the ATPase activity with young and old organisms paralleled the results of microscopic examination, in showing that in young and rapidly growing organisms the changes which preceded lysis took place rapidly, whereas in the older organisms this process became progressively slower (Table 7).

Table 8. *The chemical composition of fractions of Clostridium sporogenes*

Preparations of the solid residues of cells crushed in the Hughes press and after autolysis were prepared by centrifugation for 20 min at 30000*g* and washing twice in 0·2M-tris + maleate buffer, pH 7·0. Dry weights were determined on samples. Protein and other constituents were determined as follows. Protein (Lowry *et al.* 1951), carbohydrate (Dische & Shettles, 1948), DNA (Burton, 1956), RNA (Mejbaum, 1939). Values are expressed in terms of dry wt. of organisms after subtracting the contribution due to the suspending medium.

Sample	Protein (mg./mg. dry wt.)	RNA (μmole ribose/mg. dry wt.)	DNA (μmole deoxyribose/ mg. dry wt.)	Total carbohydrate (mg./mg. dry wt.)
Whole organisms	0·32	0·10	—	0·12
Solid residue (Hughes press)	0·25	0·10	0·17	0·063
Supernatant (Hughes press)	0·60	0·49	0·30	0·039
Solid residue (autolysis in buffer)	0·30	0·08	0·07	0·019
Supernatant (autolysed cells)	0·50	0·47	0·20	0·037

Chemical analysis of cell fractions

The solid and supernatant fractions from cell-wall membranes prepared in the Hughes press and those prepared by lysis appeared similar in the chemical composition of most of the fractions analysed except that the total carbohydrate in lysed preparations was much lower than those from the press (Table 8). Both solid preparations contained about 6–8 % total lipid of which the bulk was phospholipid as indicated by a figure of 3–4 % inorganic phosphate after ashing. Fractions of the lipid were esterified and examined by gas chromatography. There appeared to be no major difference in the distribution of fatty acids between the two types of preparations.

DISCUSSION

The work reported in this paper started with the observation that *Clostridium sporogenes* vegetative forms with a low initial ATPase activity increased in activity upon storage at 2° in various salt solutions. A similar but less marked increase had previously been seen with preparations of certain lactobacilli (Cole & Hughes, 1965). Experiments with crushed *C. sporogenes* and *C. welchii* confirmed that, as with all other bacteria so far tested, the ATPase was located in the protoplasmic membrane (Hughes, 1962). It seemed therefore that the increase of ATPase activity upon storage might be explained by an increase in the permeability of the membrane which is normally impermeable to ATP. Such changes can be caused by the addition of cationic detergents to whole cells (Dr V. Knivett, personal communication). However, further experiments suggest that the membrane probably remains intact until changes occur in the cell-wall which render the cell extremely sensitive to osmotic shock. This idea is supported by the finding that organisms incubated in sucrose solutions neither lyse nor increase in ATPase activity. Subsequent dilution

of such organisms in water results in immediate lysis and increase in ATPase activity. Microscopic examination showed organisms of normal appearance after incubation in sucrose but on dilution in water they emptied to produce typical empty cell walls. The ATPase and phospholipid were associated with the cell wall; for this reason the preparation is considered to be similar to cell-wall membranes prepared by disintegrating cells in the Hughes press (Hunt *et al.* 1959) or in the French press (Dr. J. Wimpenny, personal communication). The younger organisms produced rounded forms in sucrose; these lysed on dilution in water and are therefore referred to as 'spheroplasts'. Lysis associated with spheroplast formation could be explained by changes in cell-wall structures associated with the 'rigid layer' (Weidel, Frank & Martin, 1960). In the older organisms lysis however appeared not be be accompanied by such changes in the rigidity of the wall. A similar lysis without spheroplast formation has recently been reported by Collins (1964) and by Voss (1964). It is possible that this type of lysis may be due to a loosening of the bonding between the wall and the membrane postulated by McQuillen (1958) and Hughes (1962). The lysis discussed by Collins was brought about by a lytic enzyme derived from old organisms and that by Voss was due to lysozyme and EDTA. In both cases the lysis was accompanied by a loss of material from the outer envelopes, presumably the cell wall. Lysis due to deficiencies of cell-wall structures can be produced in growing bacteria by the addition of inhibitors of cell-wall synthesis to the medium (Welsch, 1958) or by the omission of essential cell-wall constituents from the medium (Toennies & Shockman, 1958). The autolysis of growing cultures may be due to lytic agents of microbial origin such as lysogenic phages (Jacob & Fuerst, 1958) and colicins (Fredericq, 1958). In stationary cell suspensions lytic enzymes such as those described by Collins (1964) may also produce lysis. The lysis reported here was much more marked when young organisms rather than older ones were incubated in the absence of growth medium. It could thus be explained by the exhaustion of some essential wall component consequent on some growth in the absence of extraneous nutrient medium, and depending on endogenous metabolism. Alternatively the action of lytic enzymes cannot altogether be ruled out despite their not being found in organisms or culture medium. The protection against lysis by EDTA and sulphydryl compounds would tend to support the idea of the second mechanism (see Repaske, 1960; Voss, 1964), since both these agents can modify lysis.

Thanks are due to the Rockefeller Foundation, U.S. National Institutes of Health and United States Air Force for grants to the Department. One of us (E.G.) thanks the NATO Fellowship Foundation for support during the work. Thanks are also due to Dr J. Wimpenny for helpful discussions and to Mrs H. Mitchell and Miss A. West for skilled assistance.

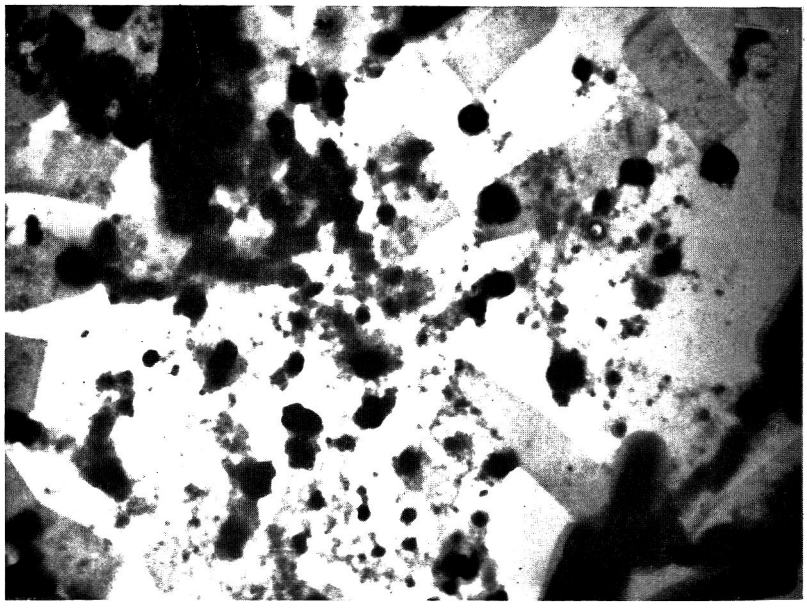

Fig. 1

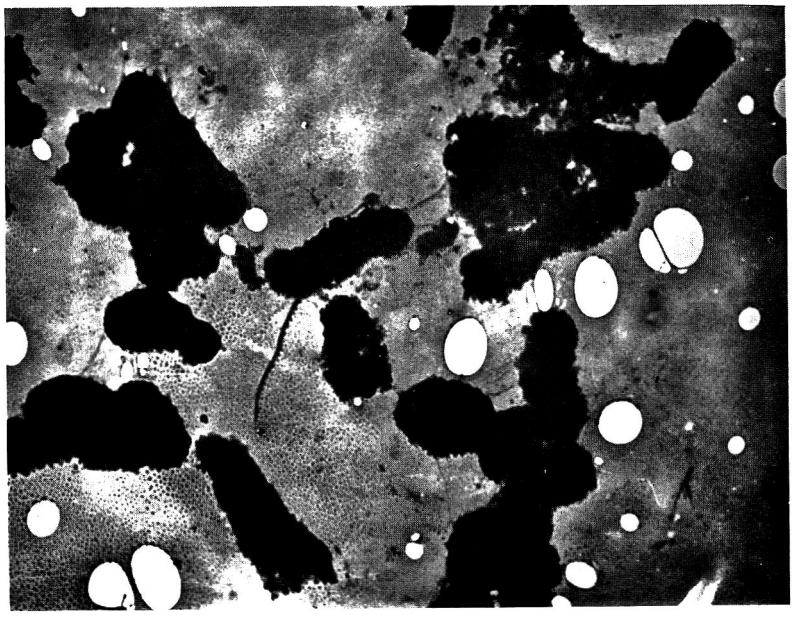

Fig. 2

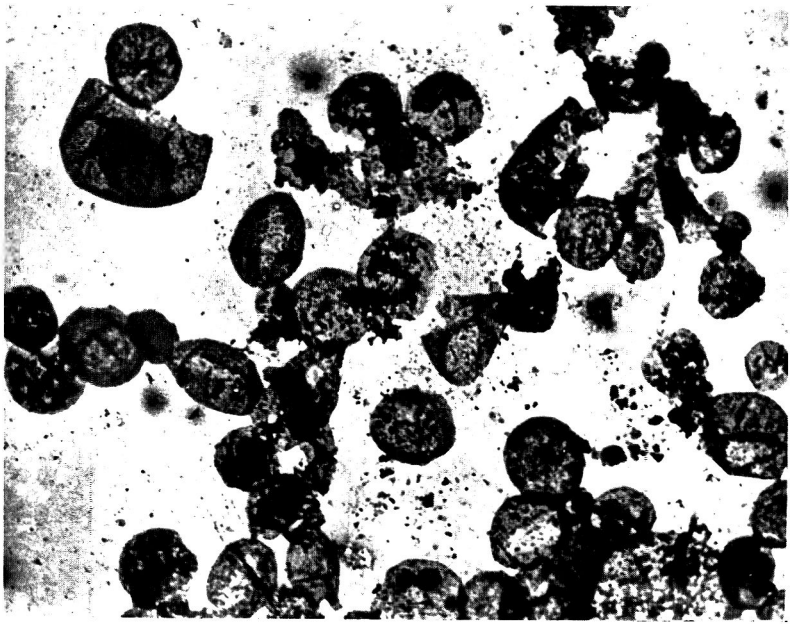

Fig. 3

Fig. 4

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EXPLANATION OF PLATES

Fig. 1. Empty cells formed by diluting cells. Cells were grown for 18 hr, collected and washed, and incubated for 18 hr in sucrose and then diluted in water. × 12,000.

Fig. 2. An early stage of lysis in young cells (8 hr growth) incubated in sucrose and showing the start of spheroplast formation. × 7,500.

Fig. 3. Empty spheroplasts, i.e. 'ghosts' formed from young cells (8 hr) incubated for 18 hr in sucrose and diluted in water. × 12,000.

Fig. 4. Cell-wall membranes from crushed cells incubated with DAase but not further treated. Shadowed with Au-Pd. × 15,000.

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Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat

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SUMMARY

The influence of Mg^{2+}-limitation, compared with carbon-limitation, on bacterial concentration, and on protein, carbohydrate, RNA and DNA contents of *Aerobacter aerogenes* cultures (grown in the chemostat at several dilution rates) was determined. In both types of culture the bacterial protein, carbohydrate and DNA contents varied slightly, and the RNA content grossly, with changes in dilution rate. Bacterial yield varied with growth rate, and to a marked degree in the Mg^{2+}-limited culture; this resulted from Mg^{2+} control of RNA synthesis. A growth-rate independent stoichiometry between RNA and Mg^{2+} was observed; 4 moles of RNA nucleotide were synthesized per mole of Mg^{2+} present in the culture. The protein and RNA distributions between cellular components varied with growth rate. The ribosomal fractions increased with increasing growth rate, as did the RNA:protein ratios in these fractions, in both cultures. Mg^{2+}-limited bacteria contained little polysaccharide; washed suspensions of such organisms synthesized polysaccharide from glycerol at a low rate as compared with C-limited bacteria. Added Mg^{2+} stimulated polysaccharide synthesis by Mg^{2+}-limited bacteria but not by C-limited bacteria. Washed suspensions of bacteria were induced to synthesize β-galactosidase. With cultures grown at three different dilution rates, the rates of enzyme synthesis in C-limited bacteria were twice those found with Mg^{2+}-limited bacteria, though both had equal ribosome contents.

INTRODUCTION

The chemostat provides unique environmental conditions in which the concentration of bacteria is controlled by the concentration of a single component of the medium (all other nutrients being present in excess of requirement) and the growth rate is controlled by the rate of supply of this component to the culture. The 'growth-limiting' substance may be any compound or element essential for the growth of the organism; when it is an element such as magnesium, the bacteria produced must contain at least the minimum concentration of this material necessary to maintain structural integrity, and the functional ability to grow at the imposed rate. To relate observations on macromolecular organization and metabolic activity specifically to Mg^{2+}-subsaturation it is necessary to compare Mg^{2+}-limited cultures with similar cultures grown in the presence of excess Mg^{2+}. To maintain comparable conditions the growth rate must then be controlled by the concentration of some

other medium component. We chose to limit growth in the control culture by the supply of carbon source (glycerol).

Magnesium is an essential component of micro-organisms (Webb, 1949). It is known to be an integral part of ribosome structure (Tissières & Watson, 1958), to be an activator of many enzyme systems (Dixon & Webb, 1958) and to influence bacterial permeability (Brock, 1962). Sykes & Tempest (1965) studied the influence of Mg^{2+}-limitation on the physiology of a fluorescent pseudomonad and reported differences in RNA concentration, ribosome composition and metabolic activity, as compared with citrate-limited growth of the same organism. The present paper reports differences found between Mg^{2+}-limited and C (glycerol)-limited cultures of *Aerobacter aerogenes* grown at several dilution rates; some of these findings are in agreement with, and others differ from, those reported for the above Pseudomonas cultures.

METHODS

Organism. Aerobacter aerogenes (NCTC 418) was maintained and subcultured monthly on tryptic meat digest agar slopes.

Growth conditions. Organisms were grown in stirred glass chemostats of 500 ml. working volume, similar in design to that described by Elsworth, Meakin, Pirt & Capell (1956). Temperature was controlled at 37°, and the pH value of the culture automatically adjusted to $6{\cdot}5 \pm 0{\cdot}1$ by a control system similar to that described by Callow & Pirt (1956). Regulated medium flow was effected by a D.C.L. Micropump (Distillers Co. Ltd., Epsom, Surrey) and the culture volume maintained constant by an internally placed overflow tube. The culture vessels, designed by Drs Herbert & Phipps (to be published), permitted vigorous agitation and aeration of the culture; foaming was suppressed by the metered addition of an antifoam (Polyglycol-P2000; Dow Chemical Co., Midland, Michigan, U.S.A.).

Large samples of culture were collected from the overflow into ice-cooled receivers; smaller samples (less than 20 ml.) were drained directly from the culture without significantly influencing the 'steady state'.

Mg^{2+}-limited medium contained: citric acid, $1{\cdot}0 \times 10^{-3}$M; $(NH_4)_2SO_4$, $5{\cdot}0 \times 10^{-2}$M; Na_2HPO_4, $5{\cdot}0 \times 10^{-3}$M; KH_2PO_4, $4{\cdot}5 \times 10^{-2}$M; $CaCl_2$ and $FeCl_3$, each $0{\cdot}1 \times 10^{-3}$M; $MgCl_2$, $2{\cdot}5 \times 10^{-4}$M; trace amounts of Mn^{2+}, Cu^{2+} and Na_2MoO_4. Glycerol was added to final concentration 50 mg./ml. Medium was made in 20 l. volumes, with distilled water which had been passed through a mixed-bed ion-exchange resin. The final pH was $5{\cdot}2 \pm 0{\cdot}1$ and at this value precipitation of salts did not occur, either on autoclaving (121°, 30 min.) or on standing at room temperature for periods of up to 3 weeks.

C-limited medium was similar to that described above except that the Mg^{2+} concentration was increased to $1{\cdot}25 \times 10^{-3}$M, and the glycerol concentration decreased to 10 mg./ml.

Bacterial size. Bacteria were suspended in normal saline containing 0·5% (v/v) formalin and size determinations carried out microscopically, with a Dyson image-splitting eye-piece, in the manner described by Powell & Errington (1963).

Colony counts of viable bacteria. These were made by spreading 0·2 ml. volumes of a suspension of organisms, containing 300–600 viable organisms/ml. (prepared by successive dilutions in saline), on the dried surface of each of five nutrient agar plates. Colonies were counted after incubation for 16–24 hr at 37°.

Viability. Measurements were made by the slide-culture technique described by Postgate, Crumpton & Hunter (1961).

Analytical procedures. Quantities of bacteria were measured by weighing oven-dried (104°, 7 hr) washed pastes from 5·0 ml. volumes of culture. Protein was determined by the biuret reaction (after Stickland, 1951) on heat treated (100°, 5 min., N-NaOH) bacterial suspensions. Bovine serum albumin, fraction 5 (Armour Laboratories) was used as standard. Ribonucleic acid was determined by the orcinol reaction (Militzer, 1946) with yeast ribonucleic acid (Boehringer and Son) as standard. Deoxyribonucleic acid was estimated by the method of Burton (1956) with calf thymus deoxyribonucleic acid (The British Drug Houses Ltd.) as standard; polysaccharide by the method of Fales (1951) with glucose as standard.

Fractionation procedure. Cultures (1–2 l.) collected in ice-cooled receivers were centrifuged (10,000 rev./min., 20 min.) in the 6 × 250 ml. angle head (no. 69179) of a MSE High Speed 17 refrigerated centrifuge. Packed bacteria were rinsed with ice-cold buffer (0·01 M-tris + 0·001 M-magnesium acetate; pH 7·5), resuspended in buffer and again sedimented at 14,000 rev./min. in the 6 × 100 ml. angle rotor (no. 69180). Bacterial pastes were transferred to a Hughes press (Hughes, 1951), precooled to −20°, and the organisms crushed, the crushed pastes diluted with buffer (4 ml./g. frozen extract) and homogenized. Microscopical examination of the homogenate showed that the proportion of intact bacteria remaining was less than 0·1 % of the initial populations.

Homogenates were centrifuged (25,000 rev./min., 45 min.) in a no. 30 rotor of a Spinco model L ultracentrifuge. Clear supernatant fluids decanted from compact fibrous deposits were again centrifuged (40,000 rev./min., 2 hr) in a no. 40 rotor of a Spinco model L ultracentrifuge. Supernatant fractions were carefully separated from the glassy pellets and the latter resuspended in cold buffer to a measured final volume. The fibrous deposit was labelled 'debris', the final supernatant fluid 'supernatant fluid' and the glassy pellet 'ribosome' fraction.

β-Galactosidase induction and assay. β-Galactosidase was induced in aerated washed suspensions of bacteria (adjusted to about equiv. 0·8 mg. dry wt./ml. in 0·067 M-phosphate buffer (pH 6·5) at 34°, by addition of methyl β-D-thiogalactopyranoside (0·001 M final concentration). Samples, taken at 45 min. intervals, were washed free from inducer with buffer and treated with benzene (0·1 ml./4·0 ml. sample) followed by shaking for 5 min. at room temperature. The enzyme was assayed by determining *o*-nitrophenol (in alkaline solution) released from 0·002 M *o*-nitrophenylgalactoside after 15 min. at 34° in 0·067 M-phosphate buffer (pH 7·2).

RNA and DNA synthesis. Bacteria from each culture, grown at a dilution rate of 0·2 hr^{-1}, were washed by centrifugation from a Mg^{2+}-deficient medium (pH 6·5; the medium used for growth of Mg^{2+}-limited organisms but without added Mg^{2+}). Washed organisms were suspended in this medium to a final concentration equiv. 2 mg. dry wt. organisms/ml. and incubated at 37° on a reciprocating shaker. Samples (5·0 ml.) were taken at 0·5 hr intervals, washed by centrifugation from distilled water and resuspended in distilled water (5·0 ml. final volume). Samples were extracted at 4° with 0·25 N-$HClO_4$ for 0·5 hr; organisms were sedimented in the centrifuge and resuspended to a known volume in 0·5 N-$HClO_4$. After incubating the suspension at 70° for 0·5 hr, organisms were removed by centrifugation and RNA and DNA analyses carried out on the supernatant solution.

Polysaccharide synthesis. Bacteria from each culture, grown at a dilution rate of 0·2 hr^{-1} were washed by centrifugation from 0·067 M-phosphate (pH 6·5) and suspended in this buffer to a final concentration of equiv. about 2 mg. dry wt./ml. Glycerol was added to final concentration 5 mg./ml. and, where necessary, Mg^{2+} to a final concentration of 5 mM. Suspensions were incubated at 37° on a reciprocating shaker, and samples (4·0 ml.) taken at intervals of 0·5 hr. Samples were washed by suspension and centrifugation from distilled water, resuspended in distilled water to final volume 10 ml. and 0·5–1·0 ml. samples taken for analysis.

RESULTS

The influence of Mg^{2+} concentration on bacterial concentration in Mg^{2+}-limited cultures

In the chemostat the 'steady state' bacterial concentration is a function of limiting nutrient concentration, K_s and dilution rate D (Herbert, Elsworth & Telling, 1956). At a fixed dilution rate bacterial yield (g. dry wt. organism formed/g. limiting nutrient consumed) is constant. Figure 1 shows the 'steady state' concentration of organisms determined for three Mg^{2+} input concentrations. The fact that there was a linear relationship between these points showed that the cultures were limited in their growth solely by the availability of Mg^{2+}; that the line extrapolated to zero concentration indicated that the culture had an absolute Mg^{2+} requirement for growth in this simple salts medium (see Webb, 1949).

Table 1. *Variation in bacterial size and growth yield with dilution rate for Aerobacter aerogenes*

	Mg^{2+}-limited*		C-limited	
D	Cell size		Cell size	
(hr^{-1})	(μ)	Yield†	(μ)	Yield‡
0·09	—	—	—	0·373§
0·10	—	832	—	0·432
0·20	2·06 × 0·90	588	1·55 × 0·70	0·452
0·20	—	673	—	0·512
0·39	—	—	—	0·498
0·40	2·14 × 0·90	—	1·64 × 0·73	—
0·41	—	460	—	0·504
0·43	—	432	—	—
0·60	—	378	—	0·510
0·82	3·26 × 1·11	347	2·53 × 0·91	0·540

* Viability did not vary beyond the range 95–100 %, at any dilution rate, in either type of culture.

† g. bacteria/g. Mg^{2+}. ‡ g. bacteria/g. glycerol.

§ Figures not corrected for dilution by pH titrant (3–6 % medium input rate).

Influence of dilution rate on the yield constant and average cell size

Herbert (1958) reported that with C-limited chemostat cultures of *Aerobacter aerogenes* both the 'yield' and mean size were functions of the dilution rate. Table 1 shows comparable figures for Mg^{2+}-limited and C-limited cultures of this organism. In both types of culture organism size varied with changes in dilution rate but, at corresponding growth rates, Mg^{2+}-limited bacteria were, on average, the larger. In

agreement with Webb (1949) we observed few aberrant forms in these 'defined' environments. The yield varied with dilution rate in both cultures but not in a like manner. With the C-limited culture a decrease in yield at low dilution rate occurred; this was attributed by Herbert (1958) to endogenous metabolism. The reverse change in yield with dilution rate found with the Mg^{2+}-limited culture was similar to that reported by Herbert (1961) to occur with N-limited cultures of *Torula utilis*, and to be due to intracellular deposition of polysaccharide material. No excess polysaccharide was, however, detected in these Mg^{2+}-limited *A. aerogenes* bacteria (see Table 2). Postgate & Hunter (1962) reported a variation in yield with dilution rate in Mg^{2+}-limited cultures of a variant of this organism; their yields were in good agreement with the figures quoted in Table 1.

Table 2. *Gross composition of Aerobacter aerogenes grown at different dilution rates*

Values are expressed as g. component/100 g. lyophilized bacteria.

D (vol./hr)	Protein	Acid sol. material (as RNA)	RNA	DNA	Carbohydrate
		Mg^{2+}-limited			
0·1	75·0	0·66	8·75	3·59	2·17
0·1	73·6	0·72	8·00	3·43	2·48
0·2	79·0	0·64	11·50	3·83	2·59
0·2	72·3	0·98	10·55	3·49	2·19
0·4	74·7	0·94	15·00	3·15	—
0·4	70·0	1·12	15·15	3·29	2·84
0·8	69·0	1·70	16·40	2·64	—
0·8*	66·3	0·96	13·10*	2·69	4·29
		C-limited			
0·1	72·2	0·90	9·50	4·20	3·79
0·1	74·0	0·84	9·25	4·23	3·36
0·2	71·0	0·90	10·35	4·27	2·88
0·2	73·5	0·80	10·75	4·61	2·77
0·4	76·0	0·88	13·95	4·27	3·29
0·4	73·6	1·16	14·30	4·31	—
0·8	69·8	0·96	18·60	3·12	3·54
0·8	67·3	0·96	18·00	2·97	3·30

* Change in cell composition following temporary pH control failure.

Variations in macromolecular composition with changes in growth rate

The gross macromolecular composition of the bacteria, grown at four dilution rates, in each type of culture is detailed in Table 2. At similar dilution rates minor differences were apparent between the two cultures. Over much of the range of dilution rates Mg^{2+}-limited bacteria contained, on a dry-weight basis, less polysaccharide and DNA than C-limited organisms, but considering the differences in average bacterial size (Table 1), the contents per bacterium were similar.

The RNA content of bacteria from each culture was a function of growth rate. This has also been found with sulphur-, phosphorus-, and nitrogen-limited cultures of this organism (Dr D. Herbert, personal communication). Although only small differences were apparent between cellular RNA contents at corresponding dilution rates, the influence of dilution rate on culture-RNA content was strikingly different.

Fig. 2 shows that whereas in C-limited environments the culture-RNA concentration was a function of dilution rate, it was independent of this parameter when the Mg^{2+} concentration limited growth. In this latter case the increase in cellular-RNA in faster growing cultures was offset by a decrease in the bacterial concentration (Table 3) such that the culture-RNA concentration remained constant over the whole range of dilution rates examined. This finding suggests that Mg^{2+} regulated RNA synthesis.

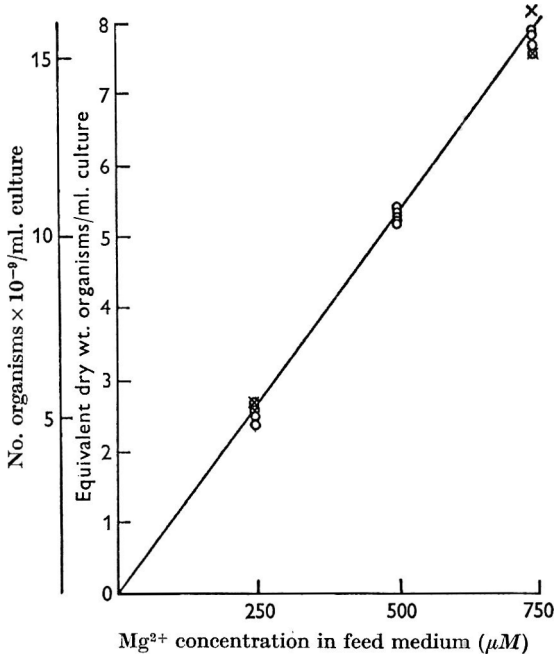

Fig. 1

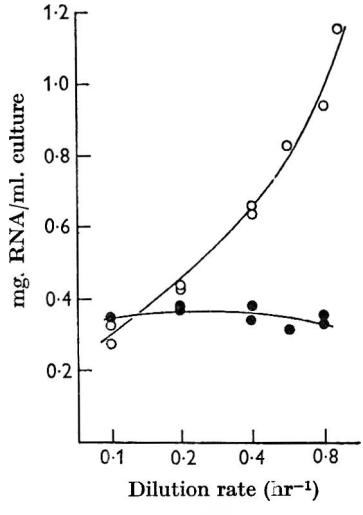

Fig. 2

Fig. 1. Bacterial concentration versus Mg^{2+} concentration in Mg^{2+}-limited cultures of *Aerobacter aerogenes* growing in the chemostat at a dilution rate of 0·4 hr^{-1}. ○, Equivalent dry wt. organism/ml. culture; ×, number of viable bacteria/ml. culture.

Fig. 2. Variation in culture-RNA concentration with dilution rate in Mg^{2+}-limited, and in C-limited, cultures of *Aerobacter aerogenes*. ●, RNA concentration (mg./ml. culture) in the Mg^{2+}-limited culture; ○, RNA concentration (mg./ml. culture) in the C-limited culture.

With an input Mg^{2+} concentration of 6 μg./ml. in the Mg^{2+}-limited culture, the culture-RNA concentration was 350 ± 40 μg./ml. over a range of dilution rates from 0·10 to 0·82 hr^{-1}. Assuming nearly complete Mg^{2+} utilization, the molar RNA nucleotide:Mg^{2+} ratio was approximately 4. As some intracellular Mg^{2+} must have been serving a metabolic role, and was therefore not available for ribonucleoprotein stabilization, the ribosomal RNA nucleotide:Mg^{2+} ratio must approach 5 which, according to Edelman, Ts'o & Vinograd (1960), is the limit for stability *in vitro* of 80S (rabbit reticulocyte) ribosomes. The figures for DNA content (Tables 2, 3) indicate that a constant DNA:Mg^{2+} ratio did not hold in Mg^{2+}-limited bacteria.

RNA and DNA synthesis by organisms incubated in a Mg^{2+}-deficient medium

The stoichiometry between culture Mg^{2+} and RNA contents suggests that Mg^{2+} controls RNA synthesis. In partial support of this was the observation (Tables 2, 3) that when a shift in the 'steady state' concentration of the bacteria occurred (following a temporary failure in the pH control system) the culture-RNA content did not vary; the cellular-RNA content changed from 16·4 to 13·1 % of the dry weight.

Table 3. *Variation in bacterial concentration, RNA and DNA concentration with changes in dilution rate for Aerobacter aerogenes*

D (vol./hr)	Dry wt.	RNA		DNA
		Cold Acid Sol.	Cold Acid Insol.	
		mg./ml. culture		
		Mg^{2+}-limited		
0·10	4·99	0·038	0·334	—
0·20	3·53	0·050	0·388	0·092
0·20	4·04	0·030	0·374	0·103
0·41	2·76	0·035	0·346	0·080
0·43	2·59	0·035	0·384	—
0·60	2·27	0·039	0·314	—
0·82	2·08	0·036	0·340	0·055
0·79	2·56*	0·035	0·362	—
		C-limited		
0·09	3·73	0·024	0·266	—
0·10	4·32	0·043	0·314	—
0·20	4·52	0·048	0·430	0·151
0·20	5·12	0·036	0·436	0·158
0·39	4·98	0·060	0·616	0·130
0·40	5·04	0·062	0·636	—
0·60	5·10	0·056	0·826	—
0·81	5·44	0·066	0·940	—
0·82	5·40	0·077	1·154	—

* Unexplainable shift in steady state bacterial concentration following trouble with pH control. See Table 2 and text.

A further indication that RNA synthesis was controlled by the availability of Mg^{2+} was obtained from observations of RNA and DNA synthesis by washed suspensions of Mg^{2+}-limited organisms and of C-limited organisms, incubated in a Mg^{2+}-deficient medium (Fig. 3). C-limited bacteria, which were found on analysis to contain twice the Mg^{2+} concentration of Mg^{2+}-limited organisms grown at the same dilution rate, synthesized considerably more RNA than did Mg^{2+}-limited organisms. Some RNA was, however, synthesized by the latter; this may have reflected Mg^{2+} contamination of the medium or utilization of residual unbound Mg^{2+} in the organisms. In contrast to this finding little difference was found between the rates of DNA synthesis in the two cultures (Fig. 3).

Variations in cell protein and RNA distribution with changes in dilution rate

Protein and RNA contents of Mg^{2+}-limited and C-limited bacteria, grown at the same dilution rates, were similar. In view, however, of the strong evidence that Mg^{2+} stabilizes ribosomal structures *in vitro* (Edelman *et al.* 1960; Ohtaka & Uchida, 1963) and *in vivo* (McCarthy, 1962), it is pertinent to inquire whether the RNA is distributed in a like manner in bacteria from the two kinds of cultures examined here. Detailed in Table 4 are the distribution patterns for protein and RNA in the two types of culture, grown at four different dilution rates (D). At corresponding values of D the RNA distribution patterns were very similar. Differences in protein distribution were apparent, particularly with respect to the 'debris' fraction. These, and the variation in 'debris' protein with dilution rate, must be viewed against the fact that bacterial size (and hence surface area:volume ratio) is a function of growth-limiting condition and growth rate.

Table 4. *Distribution of protein and RNA between fractions derived from Hughes press disintegrates of Aerobacter aerogenes*

	Protein* mg./ml. homog. protein				RNA* mg./ml. homog. protein			
D (vol./hr)	Homogenate	Debris	Supernatant fluid	Ribosome	Homogenate	Debris	Supernatant fluid	Ribosome
				Mg^{2+}-limited				
0·1	1·0	0·41	0·46	0·13	0·12	0·04	0·03	0·05
0·2	1·0	0·40	0·47	0·13	0·15	0·06	0·04	0·05
0·4	1·0	0·35	0·50	0·15	0·19	0·05	0·06	0·08
0·8	1·0	0·33	0·49	0·18	0·25	0·06	0·07	0·12
				C-limited				
0·1	1·0	0·38	0·48	0·14	0·13	0·04	0·04	0·05
0·2	1·0	0·43	0·42	0·14	0·16	0·06	0·04	0·06
0·4	1·0	0·40	0·46	0·14	0·18	0·06	0·05	0·07
0·8	1·0	0·38	0·44	0·18	0·28	0·08	0·07	0·13

* The figures quoted in this table are mean values from two cultures harvested and processed on separate days.

In agreement with the observations of Ecker & Schaechter (1963) we found the ribosome concentration to be a function of growth rate. It is evident from Table 4, however, that the relative RNA and protein contributions to the ribosomal fractions varied with dilution rate. At a dilution rate of 0·2 hr^{-1} the RNA:protein ratios were 0·43 and 0·38, respectively, for preparations from C-limited and Mg-limited bacteria, increasing to values of 0·72 and 0·67, respectively, at a dilution rate of 0·8 hr^{-1}.

Purification of the ribosomal fractions from C-limited and Mg^{2+}-limited bacteria, grown at a dilution rate of 0·2 hr^{-1}, by sedimentation for 2 hr (twice) from 0·01 M-tris + 0·001 M-magnesium acetate (pH 7·5) at 35,000 rev./min. in the SW.39 rotor of a Spinco preparative ultracentrifuge, yielded preparations which on analysis were found to contain RNA and protein in the ratios of 0·80 and 0·71, respectively. During purification 49·5% of the initial ribosomal RNA and 74·5% of the initial ribosomal protein was not recovered in the C-limited preparation; corresponding

figures for a Mg^{2+}-limited preparation were 39·5 % RNA and 68·0 % protein. Part of these losses can be attributed to removal of occluded material, but breakdown of ribosomal structures was indicated by the appearance of insoluble material on re-suspension of the ribosomal pellets.

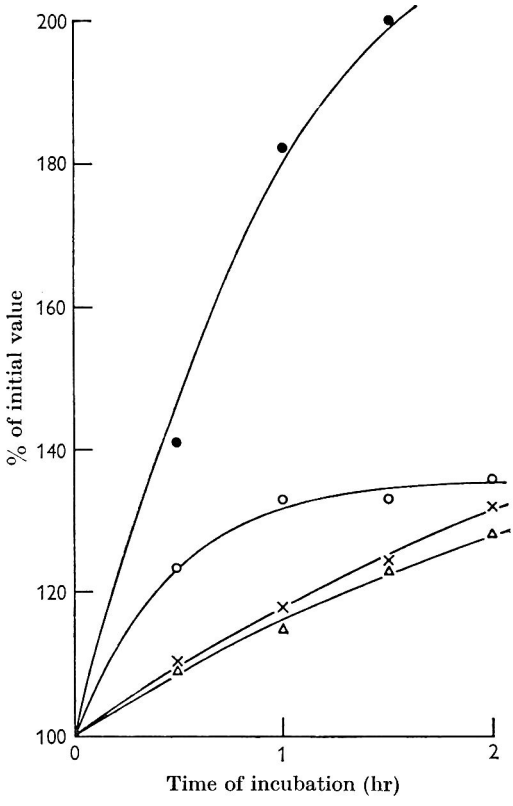

Fig. 3

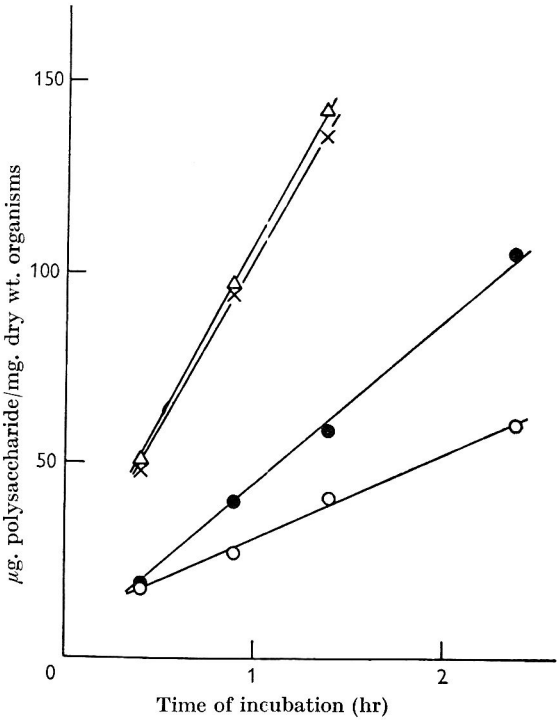

Fig. 4

Fig. 3. RNA and DNA synthesis by Mg^{2+}-limited and by C-limited *Aerobacter aerogenes* organisms incubated in a Mg^{2+}-deficient medium at 37° and pH 6·5. ○, RNA synthesis and ×, DNA synthesis by Mg^{2+}-limited organisms; ●, RNA synthesis and △, DNA synthesis by C-limited organisms.

Fig. 4. Polysaccharide synthesis by washed suspensions of *Aerobacter aerogenes*. Organisms suspended in 0·067 M-phosphate (pH 6·5), containing 0·5 % (w/v) glycerol. Mg^{2+}-limited organisms were incubated (○) in the absence of, and (●) in the presence of 5 mM-Mg^{2+}. C-limited organisms were incubated (×) in the absence of, and (△) in the presence of 5 mM-Mg^{2+}.

Polysaccharide synthesis in Mg^{2+}-limited bacteria

Nitrogen-limited or sulphur-limited bacteria grown at low dilution rates in a glycerol salts medium contained quantities of polysaccharide material in excess of that found in bacteria from a carbon-limited culture (Dr D. Herbert, personal communication). This suggests that, at these dilution rates, polysaccharide synthesis resulted solely from excess glycerol in the environment; the absence of a repressor substance does not seem to have been an influencing factor.

In Table 2 it is apparent that Mg^{2+}-limited bacteria grown at low dilution rates

contained less polysaccharide than C-limited bacteria, indicating an impaired ability to synthesize this material. Observations on the rates of polysaccharide synthesis by washed suspensions of C-limited and Mg^{2+}-limited bacteria (Fig. 4) showed that without added Mg^{2+} the former synthesized polysaccharide from glycerol at about four times the rate of the latter (87 *versus* 18 μg./mg. bacterial dry wt./hr, at 37°). Addition of Mg^{2+} to the suspending medium had little effect with the C-limited bacteria, but markedly stimulated synthesis in bacteria from the Mg^{2+}-limited culture. The rate of polysaccharide synthesis was still, however, twice as high in bacteria from the C-limited culture (92 *versus* 44 μg./mg. bacterial dry wt./hr at 37°). It would seem, therefore, that the low polysaccharide content of Mg^{2+}-limited bacteria resulted from both decreased enzyme biosynthesis and a lack of excess free Mg^{2+}.

Table 5. *Initial rates of synthesis by Aerobacter aerogenes of β-galactosidase after exposure to methyl β-D-thiogalactopyranoside and incubation at* 34°

Limiting component	Dilution rate (vol./hr) 0·1	0·4	0·8
	Rate of synthesis*		
Carbon	0·12	0·21	0·21
Mg^{2+}	0·06	0·12	0·09

* Rates expressed as change in extinction E_{420}/mg. dry wt. bacteria/hr.

Rates of β-galactosidase synthesis

Mg^{2+}-limited bacteria contained sufficient Mg^{2+} to maintain the functional ability necessary for growth at the imposed rate. From Table 2 it follows that, at similar dilution rates, the rates of protein synthesis in C-limited and Mg^{2+}-limited cultures were identical. Protein synthesis in C-limited bacteria must be limited by the availability of monomers, whereas this may well not be true for Mg^{2+}-limited bacteria where nitrogen-, carbon-, and energy-sources are readily available. It seems probable, therefore, that, though in the growing cultures the rates of protein synthesis were similar, their biosynthetic potentials differed.

Determination of rates of induced enzyme synthesis may be used as a measure of protein synthesizing ability. Table 5 lists the initial rates of β-galactosidase synthesis in bacteria from C-limited and from Mg^{2+}-limited cultures following exposure to the gratuitous inducer methyl β-D-thiogalactopyranoside. It is apparent that β-galactosidase synthesis in C-limited bacteria was more rapid than in Mg^{2+}-limited bacteria grown at the same rate. The lower biosynthetic rate observed in the latter did not appear to result from lack of free Mg^{2+}; addition of $MgCl_2$ (1 mM) to the induction medium did not effect a significant increase in the rate of enzyme synthesis.

DISCUSSION

Whereas Mg^{2+}-limited *Pseudomonas fluorescens* organisms differed from C-limited *P. fluorescens* in their total RNA content and ribosome composition (Sykes & Tempest, 1965), no consistent difference in RNA content was observed between Mg^{2+}-limited and C-limited *Aerobacter aerogenes* cultures. The difference in the RNA:protein ratios found between purified ribosome preparations from the Mg^{2+}-

limited and C-limited *A. aerogenes* (grown at a dilution rate of 0·2 hr^{-1}) was much smaller than that reported for *P. fluorescens*. In agreement with the observations of Sykes & Tempest (1965) we find that the RNA:protein ratios of the crude ribosome fractions of *A. aerogenes* increased with increasing growth rate. This change in composition may reflect a change in the basic ribosome structure, but might result from a variation in the degree of messenger-RNA and/or nascent protein associated with the ribosomes. Furthermore, a varying susceptibility to ribonuclease attack, or variation in the activity of this enzyme with growth rate (see Brody, 1957) might be contributing factors. Although the available evidence does not permit a decision between these possibilities it is clear from the present investigation, and the observations of others (Wade & Robinson, 1963; Lucas, Schuurs & Simpson, 1964) that both growth conditions and genetic differences can influence ribosome content, composition and activity.

The finding of a linear relationship between bacterial dry weight and input Mg^{2+} concentration which extrapolates to zero (Fig. 1) clearly shows that magnesium is essential for growth of *Aerobacter aerogenes* in a simple salts environment (see Webb, 1949) and suggests quantitative uptake of Mg^{2+} from the environment. The RNA, DNA and protein contents of Mg^{2+}-limited *A. aerogenes* did not appear to differ significantly from C-limited organisms grown at corresponding rates (Table 2) nor, taking into account the increased polysaccharide contents, from similar cultures grown in NH_4^+-limiting or SO_4^{2-}-limiting environments (Dr D. Herbert, personal communication). This is particularly evident with the bacterial RNA contents, which vary markedly with growth rate. In Mg^{2+}-limited *A. aerogenes* the bacterial yield (g. dry wt. bacteria formed/g. Mg^{2+}) is also a function of growth rate but the culture-RNA content (g. RNA/ml. culture) is independent of this parameter. Clearly this stoichiometry between culture-RNA and Mg^{2+} suggests Mg^{2+} control of RNA synthesis. This hypothesis is supported by the finding (Fig. 3) that washed suspensions of Mg^{2+}-limited *A. aerogenes* are less able to synthesize RNA than similar suspensions of C-limited organisms. It is not obvious how Mg^{2+} can control RNA synthesis but it is apparent from Table 4 that both ribosomal-RNA and soluble-RNA were equally subject to this control in our experiments.

Kennell & Magasanik (1962) showed that *Aerobacter aerogenes* organisms depleted in ribosomes by incubation in a Mg^{2+}-deficient medium, synthesized inducible enzymes at rates proportional to their concentration of ribosomal RNA. Mg^{2+}-limited *A. aerogenes* (as opposed to the Mg^{2+} depleted organisms used by these workers) did not have a decreased ribosomal content compared with C-limited bacteria grown at the same rate (see also Sykes & Tempest, 1965), yet were still only able to form β-galactosidase at a decreased rate (Table 5). This suggests differences in the activity of the ribosomes; but other factors (e.g. differences in protein turnover rates) might contribute to the overall result. The absence of significant amounts of polysaccharide from Mg^{2+}-limited bacteria was surprising; presumably the intracellular 'free' Mg^{2+} content was so low that Mg^{2+}-requiring enzymes of the glycogen biosynthetic pathway were virtually inactivated. The observation that Mg^{2+}-limited bacteria did not synthesize polysaccharide at a rate comparable to that of C-limited organisms even in the presence of excess free Mg^{2+} indicates quantitative differences in the enzymic constitution of these two types of *A. aerogenes*.

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Inhibition of Microbial Multiplication by Hypocholesteraemic Compounds

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SUMMARY

The hypocholesteraemic compounds benzmalecene and triparanol inhibited multiplication of certain bacteria (especially Gram-positive organisms), protozoa, algae and ascomycetes but not other higher fungi. Oleic acid (or methyl oleate) annulled the inhibition in bacteria, algae, protozoa and a yeast.

INTRODUCTION

Certain hypocholesteraemic compounds, e.g. benzmalecene and triparanol, which interfere with sterol synthesis in mammals (Holmes & Di Tullio, 1962) have been observed to inhibit protozoan multiplication (Aaronson, Bensky, Shifrine & Baker, 1962) and respiration (Aaronson, 1964*a*), and *Rhodopseudomonas palustris* multiplication (Aaronson, 1964*b*). The inhibitions of microbial multiplication were annulled best by unsaturated fatty acids; sterols and sterol precursors were less effective or were ineffective. Presumably the hypocholesteraemic compounds interfere with unsaturated fatty acid metabolism in the micro-organisms, rather than with sterol metabolism as in mammals.

METHODS

Micro-organisms from the collection of Haskins Laboratories and the Biology Department, Queens College, New York, were used. For disc assays, filter-paper discs (12·7 mm. diam.) were soaked in solutions of the test compound, dried and autoclaved. Various agar media appropriate for the growth of the micro-organisms, e.g. nutrient agar for the bacteria, and Sabouraud's glucose agar for the fungi, were seeded with test micro-organisms. Cultures were incubated at 25° for 5–10 days. In some experiments chemically defined liquid media were used appropriate to the protozoan or alga. Benzmalecene (BM; *N*-[1-methyl-2,3-di-(*p*-chlorophenyl)-propyl]maleamic acid) was supplied by Dr D. Hendlin (Merck, Sharp and Dohme Laboratories, Rahway, N.J.). Triparanol (TR; 1-[*p*-(*β*-diethylaminoethoxy)-phenyl]-1-(*p*-tolyl)-2-*p*-chlorophenyl ethanol) was supplied by Dr F. J. Murray (W. S. Merrell, Cincinnati, Ohio). Benzmalecene was dissolved in dilute alkali, and triparanol in 95% (v/v) ethanol in water. Oleic acid (99% pure by gas–liquid chromatography) was bought from the Hormel Institute, Austin, Minnesota. The other chemicals were of a commercial grade.

Table 1. *Inhibition of micro-organisms by hypocholesteraemic compounds*

Key:
[1] Bacteria showed zone of inhibition around discs soaked in 0·1 % or 1·0 % (w/v) concentration of inhibitor.
* Inhibited on nutrient agar plates but not in defined liquid medium.
† Inhibited on nutrient agar plates and in defined liquid medium.
‡ Results of M. Shifrine.
§ Results of G. G. Holz, Jr.

Schizomycetes	Benz-malecene[1]	Tri-paranol[1]
Gram-positive		
Bacillus cereus	+	+
B. megatherium	+	+
B. mesentericus	+	+
B. mycoides	+	+
Brevibacterium linens	+	+
Corynebacterium linens	+	+
C. pointsettiae	+	+
C. pseudodiphtheriticum	+	+
C. xerosus	+	+
C. bovis	+	+
Lactobacillus casei	−	−
L. plantarum	.	+§
Micrococcus luteus	+	+
M. lysodeikticus	+	+
M. roseus	+	+
M. sodonensis	+	+
Sarcina flava	+	+
S. lutea	+	+
Staphylococcus albus	+	+
S. aureus	+	+
S. citreus	+	+
Mycobacterium lacticola	+	+
M. smegmatis	+	+
M. phlei	.	+§
Gram-negative		
Aerobacter aerogenes	−*	−*
Agrobacterium tumefaciens	.	+§
Escherichia coli	−†	−†
Klebsiella pneumoniae	−	−
Paracolobactrum intermedium	−	−
Proteus mirabilis	−	−
P. vulgaris	−	−
Pseudomonas aeruginosa	−	−
Salmonella gallinarum	−	−
Serratia marcescens	−	−
Rhodopseudomonas palustris	+	+

	Benz-malecene[1]	Tri-paranol[1]
Ascomycetes		
Dipodascus uninucleatus	.	+‡
Endomycopsis javanensis	.	+‡
Lipomyces starkey	.	+‡
Penicillium chrysogenum	.	+§
Pichia fermentans	.	+‡
Rhodotorula marina	.	+‡
Saccharomyces cerevisiae	+	+
S. rosei	.	+‡
Saccharomycopsis guttulata	.	+‡
Schizosaccharomyces pombe	.	+‡
Sordaria sp.	+	+
Torulopsis utilis	.	+§
Phycomycetes		
Phycomyces sp.	−	−
Rhizopus nigricans	−	−
Basidomycetes		
Sporobolomyces sp.	.	−‡
Algae		
Coccochloris elabens (Cyanophyta)	+	+
Chlorella pyrenoidosa (Chlorophyta)	.	+§
Protozoa		
Euglena gracilis	+	+
Ochromonas danica	+	+
Tetrahymena pyriformis	+	+
T. corlissi	.	+§

RESULTS

Almost all the Gram-positive bacteria and almost none of the Gram-negative bacteria tested were inhibited by BM and TR (Table 1). Multiplication of several algae, protozoa and ascomycetes but not phycomycetes or a basidiomycete was also inhibited (Table 1). The Gram-positive mycobacteria were most sensitive to BM and TR by plate assay (Table 2).

Inhibition by BM and TR of the multiplication of a bacterium *Corynebacterium bovis*, a blue-green alga *Coccochloris elabens*, and a yeast *Saccharomyces cerevisiae*,

Table 2. *Comparison of the antimicrobial activity of benzmalecene and triparanol on several bacteria*

The bacteria were grown on nutrient agar plates.

	Concentration of disc (%)					
	0·01	0·1	1·0	0·01	0·1	1·0
	Diameter of inhibition zone (cm.)					
	Benzmalecene			Triparanol		
Organism						
Mycobacterium lacticola	0	4	3	3	4	9
M. smegmatis	0	3	5	4	8	9
Bacillus mycoides	0	1	5	0	0	4
B. mesentericus	0	0	3	0	0	3
Staphylococcus albus	0	0	3	0	0	2
S. citreus	0	0	2	0	0	2
Micrococcus luteus	0	1	2	0	5	5
M. roseus	0	0	3	0	1	4
Sarcina flava	0	0	2	0	0	4
S. lutea	0	2	3	0	0	4
Corynebacterium poinsettiae	0	0	2	0	0	3
C. xerosus	0	0	3	0	0	2
Escherichia coli	0	0	0	0	0	0
Klebsiella pneumoniae	0	0	0	0	0	0
Proteus vulgaris	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0

Table 3. *Annulment of benzmalecene and triparanol inhibition of growth by lipids*

	Inhibition of multiplication by benzmalecene and triparanol annulled by		
	Oleic acid	Ergosterol	Squalene
Sterols not synthesized			
Bacteria			
Corynebacterium bovis	+ + +	+ +	+
Rhodopseudomonas palustris	+ + +	+ +	+
Blue-green alga			
Coccochloris elabens	+ + +	+ +	+
Protozoon			
Tetrahymena pyriformis	+	+	−
Sterols synthesized			
Ascomycete			
Saccharomyces cerevisiae	+ + +	+ +	+
Protozoon			
Ochromonas danica	+ + +	−	−

Key: + + +, most active; + +, less active; +, least active; −, not active.

was annulled by oleic acid (or methyl oleate), ergosterol, and squalene in that order, as was also inhibition of the bacterium *Rhodopseudomonas palustris* (Aaronson, 1964*b*). Saturated fatty acids were ineffective. Inhibition induced by BM and TR in micro-organisms was annulled best by oleic acid (Table 3).

DISCUSSION

The difference in sensitivity between the Gram-positive and the Gram-negative bacteria examined may be associated with the difference in lipid content of bacteria:

0·3% in Gram-positive bacteria and 11–22% in Gram-negative (Salton, 1960). The sensitivity of the ascomycetes and the insensitivity of the other fungi tested draw attention to the mounting evidence for deep-seated differences between the ascomycetes and eumycophyta. Bergh, Webb & McArthur (1964) suggested that the Gram reaction may be associated with the resistance of the lipids of Gram-positive organisms to extraction by polar solvents. Ascomycetes are Gram-positive and like the Gram-positive bacteria were inhibited by benzmalecene and triparanol, both compounds known to affect lipid metabolism in animals. The annulment of BM and TR inhibition of microbial multiplication by oleic acid, rather than by saturated fatty acids, sterols or sterol precursors, suggests that lipid metabolism in the microorganisms tested differs from lipid metabolism in mammals. In mammals BM and TR inhibit primarily sterol synthesis (Holmes & Di Tullio, 1962) and fatty acid synthesis is only secondarily affected (Garattini *et al.* 1961). The hypocholesteraemic compound triparanol has a low toxicity for humans even in high concentrations (Furman & Robinson, 1961) although side-effects have been noted (Steinberg, 1962; Achor, Christensen, Berge & Mason, 1963; Roe, 1964). The sensitivity of the Gram-positive bacteria tested, notably the *Mycobacterium* species, to triparanol and benzmalecene may prove useful in the design of a new group of chemotherapeutic agents.

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Note added in proof

Since writing this paper, the author's attention has been drawn to Smith, R.F., Shay, D. E. and Doorenbas, N. J. (1963) 'Antimicrobiol action of nitrogen-containing steroids' *J. Bact.* **85**, 1295, who state that nitrogen-containing steroids, compounds resembling certain hypocholesteremic compounds i.e. azacholestanol, inhibited the multiplication of certain yeasts, moulds, actinomycetes, and Gram-positive bacteria but not most Gram-negative bacteria tested.

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Depolymerases for Bacterial Exopolysaccharides obtained from Phage-Infected Bacteria

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SUMMARY

Several bacteriophages have been isolated which, in association with the host bacteria, produce enzymes that depolymerize the exopolysaccharide of *Escherichia coli* K12 and other slime polysaccharides of the same chemical type. Chemical analyses show the similarity of the polysaccharides produced by *E. coli* K12, by other *E. coli* strains and by *Aerobacter cloacae* NCTC 5920: all contain 28–33 % fucose, 16–19 % glucose, 25–28 % galactose, 14–22 % glucuronic acid. The action of the depolymerizing enzymes greatly decreases the viscosity of the polysaccharide solutions but does not liberate any fragments of low molecular weight. Partial purification of the enzymes was achieved by ammonium sulphate precipitation and chromatography on DEAE-cellulose. The enzymes are active against exopolysaccharides produced by bacteria in which the phages are unable to multiply. Evidence is presented to show that the structural genes for enzyme production are located on the phage genome rather than on the bacterial genome. One of the enzyme systems was unusual in that it was only produced following phage infection and lysis of mucoid host strains. Its production was induced by the polysaccharide substrate.

INTRODUCTION

In studying the composition and structure of bacterial and other polysaccharides the use of depolymerizing enzymes can provide a useful adjunct to chemical methods. Enzymes capable of hydrolysing bacterial exopolysaccharides have been obtained mainly from bacterial species isolated by the elective culture technique. Thus Dubos & Avery (1931) obtained an organism ('*Bacillus vulgatus*') from which an enzyme that decomposed the capsular polysaccharide of Pneumococcus type III was obtained. Further studies on this, or a very similar system, proved that the enzyme involved was inducible (Torriani & Pappenheimer, 1962). An alternative source for the isolation of such depolymerases is a bacterium + virulent bacteriophage system. The possibility of obtaining exopolysaccharide depolymerases from such a source was shown by Adams & Park (1956) who thus isolated an enzyme active against the polysaccharide of *Klebsiella pneumoniae* B. More recently, bacteriophages which possess enzymes able to break down polysaccharides of *Azotobacter vinelandii* have been isolated (Eklund & Wyss, 1962). In our own laboratory enzymes which depolymerize the slime polysaccharide of the A3 strain of *Klebsiella aerogenes* type 54 have been obtained from bacteriophage-infected cultures (J. M. Macgregor and J. F. Wilkinson, unpublished results). In an attempt to study the structure of the extracellular slime polysaccharide produced by mucoid strains of

Escherichia coli K12, enzymes were obtained from phage-infected bacteria. The isolation, partial purification and some properties of these enzymes are now described. A preliminary account of part of this work has already appeared (Sutherland & Wilkinson, 1964).

METHODS

Bacteria used. Aerobacter cloacae NCTC 5920 was obtained from the National Collection of Type Cultures, Colindale, London. *Escherichia coli* s23 is a gal⁻ auxotrophic mutant strain of K12 and was received from Professor E. A. Adelberg (Department of Microbiology, Yale University) as strain AB739. *E. coli* s53 was a highly mucoid variant of strain K12 obtained by Dr E. C. R. Reeve (Department of Animal Genetics, Edinburgh University) during screening of mutants for chloramphenicol resistance. *E. coli* s53c was isolated as a resistant variant of s53 during sensitivity tests with non-depolymerase-producing phages. *E. coli* s17 was a mucoid strain obtained from Professor J. P. Duguid (Bacteriology Department, St Andrews University).

Serological typing of these strains at the International Escherichia Centre (Statens Serum Institut, Copenhagen) showed that all were untypable with respect to O-antigens. Strain s53 showed cross-reaction with K30 antiserum and both s53 and s23 showed H48 flagellar antigens. Another strain K12 derivative submitted at the same time had the antigenic formula O102; (K30); H48. The s17 strain could not be typed.

Media. Bacteria were normally grown in nutrient broth or on nutrient agar. When maximal exopolysaccharide production was required, a nitrogen-deficient medium was used. It had the following composition (g./l. medium): yeast extract (Oxoid), 1·0; Casamino acids (Difco, technical), 1·0; Na_2HPO_4, 10·0; KH_2PO_4, 3·0; $MgSO_4.7H_2O$, 0·2; K_2SO_4, 1·0; NaCl, 1·0; $CaCl_2$, 0·01; $FeSO_4$, 0·001. Glucose solution (20%, w/v) was autoclaved separately at 120° for 15 min. and added to the other components after sterilization to give a final concentration of 2% (w/v).

For bacteriophage production, this medium was used in liquid form in 200 ml. lots in 500 ml. Erlenmeyer flasks. The flasks were aerated by air passed into the medium through sterile Pasteur pipettes. For polysaccharide production the medium was solidified by adding 1·5% (w/v) New Zealand agar and poured into enamel trays (40 × 30 cm.) as a thick layer. The trays were fitted with fairly loose aluminium covers and after uniform inoculation from a liquid culture of bacteria, they were incubated for 3–4 days at 30°.

Bacteriophages. The strains and methods of isolation are described in the text.

Viable counts. Viable counts of bacteria and bacteriophages were made by the method of Miles & Misra (1938).

Total nitrogen was determined by the micro-Kjedahl technique. The ammonia produced was trapped in boric acid and estimated colorimetrically with Nessler reagent.

Phosphorus. The method of Fiske & SubbaRow (1925) was used.

Fucose was determined on the unhydrolyzed polysaccharides by the method of Dische & Shettles (1948).

Glucose and galactose were determined on samples after hydrolysis with N.H_2SO_4 in sealed ampoules for 8 hr. The hydrolysates were neutralized with $BaCO_3$. Glucose

was determined with glucose oxidase reagent (Boehringer GmbH., Mannheim, Germany) and galactose with galactose dehydrogenase prepared from *Pseudomonas saccharophila* (Doudoroff, 1962).

Uronic acid was determined on the unhydrolysed material by the method described by Bowness (1957).

Paper chromatography of acid hydrolysates of polysaccharides was performed in *n*-butanol + glacial acetic acid + water (4 + 1 + 5, by vol.) or in the solvent system described by Fischer & Dörfel (1955). Sugar spots were detected with alkaline silver nitrate or with aniline oxalate.

Concentration of enzyme-containing solutions. Polyethyleneglycol (mol. wt. 6000) was used according to the method of Kohn (1959).

Determination of enzyme activity. This was assayed on old cultures of *Escherichia coli* s 53 incubated at 30° for 48 hr on nitrogen-deficient medium and stored at 0° for at least 7 days before use. The enzyme preparation was diluted in a twofold series in NaCl containing phosphate buffer (0·01 M; pH 7·2). Drops from calibrated pipettes (0·02 ml.) were carefully placed on the culture surface. The plates were incubated for 2 hr at 37° and examined for dissolution of polysaccharide. After a further 16 hr at room temperature, the plates were again examined. The enzyme titre was taken to be the highest dilution which gave complete dissolution of the polysaccharide. On occasions a tube test with a 5% (w/v) gel of purified polysaccharide was made in the same manner.

Preparation of antisera. The enzyme preparations used in obtaining antisera were high titre eluates from DEAE-cellulose columns. These were injected according to the method described by Adams (1959) for the preparation of phage antisera. The activity of the antisera in inhibiting enzyme action was determined by incubating mixtures of enzyme and suitably diluted serum at 37° for 20 min., then titrating the mixture in the way described for the enzyme preparations. Controls containing normal rabbit serum were similarly treated.

RESULTS

The isolation of depolymerase-producing bacteriophages

The source of all the phages isolated was untreated sewage from Edinburgh sewage works. To each 400 ml. sample was added 200 ml. sterile broth and 300 ml. chloroform. The mixture was then shaken vigorously for several minutes in a screw-capped bottle and then allowed to stand for 3–4 hr at 0°. The clear upper layer was then used for bacteriophage isolation without further treatment.

Nutrient broth cultures (10 ml.) of the potential host bacteria were incubated overnight and then diluted 1/10 with fresh sterile broth and incubated for 1–2 hr at 37°. To this culture was added 1 ml. of the sewage preparation and the mixture was incubated overnight at 37°. The culture was centrifuged briefly at low speed to remove most of the bacteria. The supernatant fluid was added to sterile 1 oz. screw-capped vials and heated at 60° for 30 min. to kill remaining host bacteria. A series of tenfold dilutions of this material in sterile saline were made and 0·1 ml. samples from each dilution spread over nutrient agar plates which had been flooded with a culture of host bacteria and allowed to dry. After incubation for 24 hr at 37°, the plates were examined. Where discrete plaques were visible, material representing

each plaque type was removed with a sterile needle and inoculated into 1–2 hr cultures prepared as above. These cultures were then treated in exactly the same way as the initial enrichment cultures. After sterilization at 60°, a series of dilutions was again prepared and material spread on agar plates seeded with bacteria. When, after incubation, only one plaque-type was observed, it was assumed that the phage culture was pure. Large amounts of phage for stock cultures were prepared either by the soft agar layer technique (Adams, 1959) or by growth in aerated flask cultures in nitrogen-limited medium.

Host range of the isolated bacteriophages

Phage preparations (0·1 ml.) containing 10^4 plaque-forming units (p.f.u.) were added to bacterial lawns and examined after incubation for 24 hr at 37° for confluent lysis of the bacteria. Sixty strains of *Escherichia coli* and eight strains of *Aerobacter cloacae* were tested. Of the *E. coli* strains, 12 were derivatives of strain K12 and 15 of the remainder were of known serotype. The other strains were obtained from

Table 1. *The host range of isolated bacteriophages for Escherichia coli and Aerobacter cloacae*

Bacterial susceptibility tested by observation of confluent lysis of strains of *E. coli* and *A. cloacae* grown on solid medium.

		Growth on						
		E. coli strains					*A. cloacae* strains	
Phage	Original host	s23	s53	s53c	K12	Other Others	5920	Others
	Escherichia coli							
F1	s53	+	+	+	+	−	±	−
F5, 26, 27	s53	+	+	+	+	−	−	−
F6	s23	+	+	−	±	−	−	−
	Aerobacter cloacae							
F12, 13, 14	5920	−	−	−	−	−	+	−

+ = confluent lysis, − = no lysis, ± = variable.

various sources and were selected primarily for ability to produce extracellular polysaccharide, as observed by mucoid growth and negative staining with Indian ink (Duguid, 1951). The *A. cloacae* strains were all obtained from the National Collection of Type Cultures, Colindale, London. Results of these experiments are shown in Table 1, for some of the phages isolated. All the phages isolated on *E. coli* hosts were specific for *E. coli* K12 strains. They were avirulent for other strains and, with one exception, for *A. cloacae*. The results for phage F1 were variable; although it frequently showed confluent lysis on *A. cloacae* 5920, and the numbers of p.f.u. increased when grown in liquid cultures of this organism, negative results were also obtained. The phages isolated on *A. cloacae* 5920 as host, all appeared to be specific for this strain and had no effect on the other strains of this species which were tested.

Detection of depolymerase production

The production of plaques surrounded by a large halo when grown on mucoid bacterial strains is relatively common (Park, 1956). These haloes may be due to the production of exopolysaccharide depolymerase. Alternatively, Reiter & Oram (1963) suggested that they might be caused by the production of large amounts of phage-associated lysozyme. Initially we selected as possible depolymerase producers those phages which produced plaques surrounded by haloes. These comprised all the phages listed in Table 1, except phage F6. To ensure that the haloes were indeed due to depolymerase and not to lysozyme, 1 l. volumes of nitrogen-deficient liquid medium were inoculated with host bacteria and each phage species grown in it. The bacteria were removed by centrifugation and the supernatant fluid dialysed against cold running tap water for 16 hr. Each preparation was then concentrated to about 10 ml. and centrifuged at 10,000*g* to remove particulate material. The resultant supernatant fluids were then titrated for depolymerase activity. All the phages listed, except phage F6, produced enzyme in relatively large amounts. Several phages which did not produce haloes, did not produce depolymerase; phage F6 was taken as a representative of such species when bacteriophages not producing enzyme were required.

Isolation and partial purification of depolymerases

Exopolysaccharide depolymerase preparations were obtained from each phage host system, by using phage-infected bacterial cultures grown either by the soft-agar layer technique or from aerated liquid culture growing in nitrogen-limited medium. The latter method was preferable, since all the medium components could then be removed by dialysis. The whole cultures, after incubation for 8–16 hr at 37°, were centrifuged at 6000*g* in a refrigerated centrifuge; the deposit was discarded. The supernatant fluid was dialysed against cold tap water for 10 hr, then concentrated at 0–2° to about 5% of the original volume. The concentrated material was dialysed against phosphate buffer (0·02 M; pH 7·2). Initial fractionation was done by adding saturated ammonium sulphate solution to give a series of fractions between 10 and 90% saturation, the precipitate at each stage being removed by centrifugation. In all the systems studied almost all the depolymerase-active material precipitated at 40–50% saturation. This active material was dialysed against phosphate buffer (0·02 M; pH 7·2) and the dialysis residue then applied to a column of DEAE-cellulose (12 cm. × 1·4 cm.) equilibrated against the same phosphate buffer. A solution of buffer containing an increasing sodium chloride concentration was passed through the column. The enzymically-active material from the phage F1 and F5 systems, which were most extensively studied, was eluted at a sodium chloride concentration of about 0·1 M. These preparations, after concentration and dialysis against the 0·02 M-phosphate, were used without further purification.

Substrates for the enzyme(s)

Crude preparations of the enzyme or enzymes from each phage host system liquefied the exopolysaccharide produced by the host bacterium. They showed equal activity against slime of the same chemical type from other bacteria. Thus depolymerases from the *Escherichia coli* systems were active against *Aerobacter cloacae*

polysaccharide and conversely. The production of a common mucoid substance by several species of the Enterobacteriaceae has been suggested by several authors (Anderson & Rogers, 1963; Goebel, 1963). Thus the activity of the depolymerase preparations against polysaccharides from a range of bacteria was not unexpected and tends to support the views of these authors.

Escherichia coli strain s53 produced very large amounts of extracellular slime, even when grown on nutrient agar at 37°. This production was very much enhanced by growth at lower incubation temperatures in nitrogen-limited media. Slime was prepared and purified by methods used previously for the slime polysaccharides of *Klebsiella aerogenes* (Wilkinson, Dudman & Aspinall, 1955). By these methods, material was prepared from *E. coli* s53, *E. coli* s17, and *Aerobacter cloacae* 5920. In each case, paper chromatography of acid hydrolysates showed the presence of glucose, galactose, fucose, glucuronic acid and glucuronolactone in the slimes. The

Table 2. *Composition of bacterial extracellular polysaccharides from Escherichia coli and Aerobacter cloacae*

Analyses performed on the purified slime polysaccharides isolated from cultures on nitrogen-deficient solid medium.

	E. coli strains*			*A. cloacae*	
	s17	s53	K12 capsule*	5920	5920†
	Composition (%)				
Fucose	31·2	32·8	32·7	28·0	28·3
Glucose	19·5	19·6	16·3	16·0	17·0
Galactose	26·0	25·4	33·0	27·5	26·0
Glucuronic acid	20·5	18·0	17·9	13·8	22·0
Nitrogen	0·9	0·9	0	0·9	0·3
Phosphorus	0·2	0·3	0	0·2	0·2

* Results of Sapelli & Goebel (1964). † Results of Dudman & Wilkinson (1956).

composition of the polysaccharides, after correction for a water content of 15–20 %, is shown in Table 2, with earlier results for *A. cloacae* 5920 (Dudman & Wilkinson, 1956) and for the composition of polysaccharide obtained from a capsular variant of *E. coli* K12 (Sapelli & Goebel, 1964). It should be emphasized that although *E. coli* strain s53, in our hands, normally produced only slime material, occasional capsulate forms were observed during culture. The selection of such variants was by using phage F6, which by virtue of its inability to produce depolymerase, was unable to penetrate and lyse capsulate bacteria. The capsular material from *E. coli* strain s53c had the same sugar components as the slime polysaccharide when examined by hydrolysis and paper chromatography; a detailed analysis was not made. It can be seen from Table 2 that the compositions of the different preparations were very similar, the greatest difference was in the uronic acid content. However, the methods used for this analysis differed and are probably less satisfactory than those used for estimation of the other sugars. Further evidence of the similarities between the slime polysaccharides of *E. coli* strain s53 and *A. cloacae* 5920 has been obtained by a study of the oligosaccharides produced by partial acid hydrolysis (I. W. Sutherland, unpublished results).

Effect of depolymerase of phages F1 and F5 on the substrates

Partially purified depolymerases from the phage F1 and F5 systems were incubated with substrate preparations dissolved in 0·02 M-phosphate buffer (pH 7·2) for up to 96 hr at 37°. The most noticeable effect was a decrease in the viscosity of the polysaccharide solution; this was also observed when crude enzyme preparations from other depolymerase-producing phages were used. Examination of the products of enzyme action showed that there was no marked increase in the reducing value of the polysaccharide preparation, so that there was apparently no liberation of reducing sugars or oligosaccharides. This was confirmed by the absence of any detectable diffusate products or dialysis products. Paper electrophoresis and paper chromatography also did not show any products of low molecular weight. After enzymic treatment, two fractions were obtained by ethanol or acetone precipitation. About 75% of the weight of the original polysaccharide was precipitated by the addition of an equal volume of cold acetone to the solution. The remaining material stayed in solution, was not precipitated by the addition of a further two volumes of acetone; it was obtained as a viscous syrup by per-vaporation and freeze-dried. Chemical analysis did not show any marked differences in the chemical composition of these fractions from that of the original polysaccharide, although when the acetone-soluble fraction was subjected to partial acid hydrolysis there appeared to be a difference in the pattern of oligosaccharides obtained. The phage enzymes showed no activity against several partially characterized oligosaccharides isolated from partial acid hydrolysates of the polysaccharide. The enzymes were inactive against the slime polysaccharide produced by *Klebsiella aerogenes* type 54, which is known to contain glucose, fucose and glucuronic acid (Wilkinson *et al.* 1955). Conversely, depolymerases active against this substrate were inactive against *Escherichia coli* K12 polysaccharide (J. M. Macgregor and J. F. Wilkinson, unpublished results). The depolymerases thus show a substrate specificity. Whether this depends on the presence of certain repeating units in the polysaccharide substrate must await structural studies on the substrates.

It is of interest that the depolymerase specificity was not necessarily correlated to the phage specificity. Thus enzymes from *Aerobacter cloacae* + phage systems were active against the *Escherichia coli* polysaccharide and conversely. The depolymerases were also active against the polysaccharide produced by *E. coli* s17, a strain in which none of the phages was capable of multiplication.

The effect of the host bacterium on enzyme production

In the case of all the depolymerase-producing phages except F1, growth in a susceptible bacterium was accompanied by enzyme production. Phage F1, when grown on *Escherichia coli* strain s53 (slime-producing), produced depolymerase, which was also detected following its growth in *Aerobacter cloacae* 5920. However when phage F1 was grown in *E. coli* strain s23, a non-mucoid K12 derivative, no depolymerase was detected. This suggested that in the phage F1 + bacterial host system, the depolymerase was induced by the presence of the substrate, i.e. the slime polysaccharide. When purified polysaccharide from *E. coli* strain s53 was sterilized as a 1% (w/v) solution at 100° for 90 min. and added to culture medium to a final concentration of 0·5% (w/v), growth of *E. coli* strain s23 in this medium,

followed by infection with phage F1, led to depolymerase production. However, the quantity of enzyme was less than that obtained from comparable culture in *E. coli* strain s53. Addition of the acetone-soluble product of enzyme action to the medium followed by bacterial and phage culture, did not lead to depolymerase production. Attempts to show depolymerase production in the absence of phage infection were unsuccessful. Thus, ultrasonic treatment of bacteria taken at different stages of growth never gave preparations which caused dissolution of slime polysaccharide.

The effect of depolymerase on bacterial infection

Since the substrates for the phage depolymerase enzymes are normally slime polysaccharides, i.e. do not occlude the bacterial surface, it was not at first possible to determine the effect of the enzymes on infection of bacteria by phage. However, a capsulate variant of *Escherichia coli* s53, designated s53c, was obtained. This variant was resistant to phages such as F6 which did not produce depolymerase. It was susceptible to all the coliphages which produced depolymerase. Titration of the phages on susceptible hosts showed that where the phage system resulted in depolymerase production, the phage titres obtained on slime- or capsule-forming host bacteria and on non-mucoid bacteria were very similar. No plaques were observed following addition of non-depolymerase-producing phages to capsulate host bacteria. It is probable that small amounts of depolymerase, sufficient to permit entry to capsulate bacteria, were present in close association with the phage particles. This is implied from the observation that many phage preparations which contained no detectable enzyme, as determined by titration, were still able to multiply in cultures of *E. coli* s53c.

It was also found that the purified depolymerase of phages F1 or F5 had little or no effect on bacterial viability. To a culture of susceptible bacteria in broth was added 0·1 ml. of purified enzyme preparation with a titre of 256/ml. Samples were withdrawn after various periods of incubation at 37° and bacterial counts made. As a check on the sterility of the enzyme preparations, phage titrations were also made but no plaques were observed. Counts of bacterial colonies from the cultures to which depolymerase had been added differed little from control cultures without added enzyme.

Antisera to depolymerase and to host bacteria

In an attempt to determine whether the production of depolymerase was controlled by the bacterial or the bacteriophage genome, the effect of antisera on purified depolymerase preparations of phages F1 and F5 was examined. The addition of homologous antiserum resulted in complete inhibition of depolymerase activity but antiserum to phage F1 enzyme did not inhibit phage F5 depolymerase and conversely. Antisera to *Escherichia coli* s53 and to other *E. coli* strains did not inhibit any of the phage depolymerases. This would seem to indicate that production of the depolymerases is controlled by the phage genome. In gel diffusion tests in agar, a line of precipitation was obtained between the depolymerase preparation and homologous antiserum, but not with antisera to the host bacteria.

Other properties of the depolymerases

Ultracentrifugation of purified depolymerase preparations for prolonged periods at 100,000*g* in a Spinco model L preparative ultracentrifuge resulted in no deposition of enzymically active material. Sterilization by filtration through Oxoid membranes resulted in little loss of activity.

A depolymerase isolated by Adams & Park (1956) from phage-*Klebsiella aerogenes* systems was notable for the fact that the enzyme was more heat stable than was the phage with which it was associated; 10% of the enzyme activity remained after exposure to 70° for 30 min. In our enzyme systems, all measurable activity was destroyed after 5 min. at 70°. During the same time, the phage titre on a non-mucoid bacterial host decreased from $1{\cdot}8 \times 10^8$ to $2{\cdot}0 \times 10^7$. It continued to decrease thereafter until no viable phage was detectable after 45 min. at 70°. At 65°, the enzymes were also rapidly inactivated and although the phage titre was decreased there was still considerable phage activity remaining after 60 min. The results of a typical experiment with phage F1 titrated on *Escherichia coli* s23 are shown in Table 3. Exposure of the phage enzymes to pH values above 7·5 and below 6·0 also resulted in rapid loss of activity. For this reason, all heat inactivation experiments were done in 0·05 M-phosphate buffer (pH 7·0).

Table 3. *The effect of heating at* 65° *on F*1 *phage and depolymerase titre*

Samples of phage (5 ml.) held in tubes in a water bath at 65° and samples (0·1 ml.) withdrawn at intervals and titrated on bacterial lawns on nutrient agar and old mucoid lawns on nitrogen-deficient medium respectively.

Time (min.)	Phage titre p.f.u.	Depolymerase titre
0	$6{\cdot}2 \times 10^{11}$	32
15	$5{\cdot}9 \times 10^{10}$	4
30	$9{\cdot}0 \times 10^{9}$	0–2
45	$5{\cdot}0 \times 10^{9}$	0
60	$4{\cdot}3 \times 10^{9}$	0

DISCUSSION

A number of exopolysaccharide depolymerases produced by phages in association with bacteria are now known. While some properties may be common to all such systems, there are also marked differences. Thus, Adams & Park (1956) reported that a depolymerase for *Klebsiella pneumoniae* polysaccharide was produced in all susceptible hosts. Although we obtained similar results for most of the *Escherichia coli* phages isolated, there was one exception. The results with *E. coli* K12 + bacteriophage F1 system indicated that this depolymerase is probably inducible, the presence of the substrate polysaccharide being required for enzyme production. This phage F1 differed in that the other depolymerase-producing phages isolated by us caused enzyme production in mucoid and in non-mucoid bacterial hosts.

The inducibility of the F1 enzyme is of interest in deducing a role for the depolymerase. Unlike the *Klebsiella pneumoniae* or Azotobacter systems studied by Adams & Park (1956) and Eklund & Wyss (1962), respectively, the *Escherichia coli* strains used in the present study were slime-producing but non-capsulate. The bacterial surface of such organisms is not occluded by polysaccharide and the presence of

polysaccharide-decomposing enzyme would not be essential to enable a phage particle to reach and absorb to the bacterial surface. However, the presence of large aggregates of slime might be a sufficient hindrance to prevent many of the phage particles from reaching and infecting their hosts. The presence of a depolymerase would therefore confer an advantage on such phages, in that they would disperse the protecting polysaccharide and reach the bacterial surface.

Adams & Park (1956) found that the depolymerase for *Klebsiella pneumoniae* polysaccharide was in two forms. One of these was a freely diffusable protein, while the second was attached to or firmly associated with the phage particles. Our methods were not sufficiently sensitive to permit detection of the second form. Its presence would seem likely from the observation that phage preparations with no detectable enzyme were nevertheless able to infect capsulate *Escherichia coli* organisms. Thus infection of a few bacteria, followed by lysis and release of free depolymerase, might allow the second generation of phage particles to infect a high proportion of the remaining bacteria.

The question arises as to whether the structure of the depolymerase is determined by the bacterial genome or by a structural gene of the phage. Since attempts to obtain depolymerase from the uninfected host bacteria were unsuccessful, it would seem that the production of the enzyme requires the presence of host bacteria and phage. Further, the hypothesis that the effect of phage was merely to enhance the production of an existing bacterial enzyme would appear to be discounted by the serological evidence. The enzymes were only inhibited by homologous antiserum and not by antisera to other depolymerases or to the host bacteria. In other words, the antigenic specificity of the depolymerase was determined by the nature of the phage rather than the host.

The mode of action of the depolymerases remains obscure. The lack of diffusible products following enzyme action, together with the very marked decrease in viscosity, would suggest that some internal linkage is broken, although the products of this cleavage must still be relatively large. The linkage may be one which is acid-stable, since enzymic hydrolysis followed by partial acid hydrolysis of the polysaccharides showed a much more complex pattern of oligosaccharides than did acid hydrolysis alone (I. W. Sutherland, unpublished results). The depolymerase for *Klebsiella pneumoniae* polysaccharide was found by Adams & Park (1956) to yield no detectable small molecular weight products after hydrolysis, nor did it cause an increase in the reducing value of the polysaccharide preparation. Further, the hydrolysis products retained their serological specificity, indicating that the end-group sugars were probably unaffected by the enzyme.

The relative insensitivity of methods of depolymerase assay used prevent a thorough study of the kinetics of enzyme formation following phage infection of the bacteria. Such studies must await elucidation of the substrate structure and the development of improved assay methods. The substrate of these depolymerases is of interest in that it appears to be produced by *Escherichia coli* strains and also by certain other species of the Enterobacteriaceae. This confirms the suggestions of several workers that different members of the Enterobacteriaceae may produce mucoid material of similar if not identical composition. These substances have been termed M antigens (Ørskov, Ørskov, Jann & Jann, 1963) or colanic acid (Goebel, 1963). The *E. coli* polysaccharide can be produced either as a capsule or as free slime. There

was no detectable difference in the capsular and slime polysaccharides, confirming the results previously obtained using *Klebsiella aerogenes* type 54 (Dudman & Wilkinson, 1955).

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The Effect of Ethylenediaminetetra-acetic Acid on the Cell Walls of Some Gram-Negative Bacteria

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SUMMARY

A comparative survey of amino acid, amino sugar, sugar and lipid components of the cell walls of strains of *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Escherichia coli* and *Proteus mirabilis* was made. The cell walls of *P. aeruginosa* and *A. faecalis*, against which ethylenediaminetetra-acetic acid (EDTA) has a potent bactericidal action, differed from those of the other organisms principally in their sugar components and in their high content of phosphorus. EDTA at alkaline pH selectively solubilized a high proportion of the carbohydrate and phosphorus present, apparently as lipopolysaccharides, in the walls of sensitive organisms. It is suggested that metal cations and lipopolysaccharides in the cell walls of *P. aeruginosa* and *A. faecalis* may be essential to the structural integrity of these organisms.

INTRODUCTION

The ability of ethylenediaminetetra-acetic acid (EDTA) to sensitize certain Gram-negative bacteria to lysozyme was shown by Repaske (1956), who suggested (1958) that EDTA removed or combined with metal ions which were bound by the cell wall and which interfered with the formation of the lysozyme–substrate complex. This view was supported by the similar ability of the cation-exchange resin Dowex 50 (H^+) to sensitize bacteria to lysozyme and by the inhibition of lysis of sensitized bacteria by metal ions. Other workers (e.g. Salton, 1958; Noller & Hartsell, 1961*a*, *b*) considered that EDTA disorganized one of the layers, probably lipoprotein, which overlie and protect the mucopeptide substrate of lysozyme in the cell walls of Gram-negative bacteria (Weidel, Frank & Martin, 1960). Thus, the purified mucopeptides of these bacteria were completely dissolved by lysozyme without the addition of EDTA (Weidel *et al.* 1960; Mandelstam, 1962). In the cases of various pathogenic Salmonella organisms, the action of EDTA in making the mucopeptide accessible to lysozyme was found (Colobert, 1957*a*, *b*) to involve the release of lipid material, believed to come from the O antigens of the cell walls. Treatment with EDTA was used by Colobert & Creach (1960) as a step in the purification of the mucopeptide from *Salmonella typhi*. The action of EDTA was thought by Colobert (1958) and Noller & Hartsell (1961*a*, *b*) to involve a detergent-like mechanism rather than a chelating mechanism. However, the technical bulletin quoted by Grula & Hartsell (1957) and Colobert (1958) refers only to the ability of solutions of the tetrasodium salt of EDTA to dissolve grease. This property is a function of the alkalinity and buffering capacity of concentrated solutions of the salt and would not apply with the low concentrations of EDTA in buffer of about pH 8 used in potentiation of lysozyme action.

In a previous paper (Gray & Wilkinson, 1965), it was shown that EDTA had a lytic bactericidal action on *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. Evidence was obtained that the bactericidal action took place at the cell wall and involved the removal or displacement of metal ions essential to the structural integrity of the wall. Such a function was suggested for calcium ions in the cell wall of *Rhizobium trifolii* (Humphrey & Vincent, 1962; Vincent & Humphrey, 1963). If metal ions are structural components in bacterial cell walls, then the sensitivity of an organism to EDTA should be dependent on the nature and strength of the bonds formed between the metal and the metal-binding components of the cell wall. Thus, the chemical basis for the exceptional sensitivity to EDTA of *P. aeruginosa* and *A. faecalis* might be indicated by a study of the composition of their cell walls. The present paper records the results of a survey of the cell walls from organisms of various degrees of sensitivity to EDTA. The highly sensitive organisms *P. aeruginosa* and *A. faecalis*, the moderately sensitive *Escherichia coli* and the insensitive *Proteus mirabilis* were chosen for this survey (Gray & Wilkinson, 1965).

METHODS

Organisms and preparation of cell walls. Cultures of single strains of *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Escherichia coli* and *Proteus mirabilis* were grown and the cell walls of the organisms were isolated from the strains and by the methods previously described (Gray & Wilkinson, 1965). The freeze-dried wall preparations were stored at 4° and were freshly dried (P_2O_5) *in vacuo* before being analysed.

Purity of cell-wall preparations. The absence of significant cytoplasmic contamination of the preparations was indicated by the absence of electron-dense material in electron micrographs, and by the failure to detect ribose in acid hydrolysates of the cell walls. The ultraviolet spectra of aqueous suspensions of the walls showed a minor peak or inflexion at 275–280 mμ but no absorption maximum at 260 mμ, confirming the absence of polynucleotides. The consistency ($\pm 2\%$) of analyses for phosphorus and carbohydrate and the results of quantitative evaluations of infrared spectra of different batches of walls from each organism confirmed that reproducible preparations were obtained.

Quantitative analyses. Phosphorus was estimated by the method of Allen (1940), and nitrogen by a modification of the method of Umbreit, Burris & Stauffer (1957). Estimations of reducing sugar (Hanes, 1929), carbohydrate (Trevelyan & Harrison, 1952) and amino sugar (Rondle & Morgan, 1955) were made on samples of filtered neutralized hydrolysates (2 N-HCl for 2 hr at 105°) of the cell walls.

Identification of amino acids. Hydrolysates (6 N-HCl for 16 hr at 105°) of cell walls were examined by two-dimensional chromatography by using Whatman no. 1 paper and *sec*-butanol + 88% formic acid + water (75 + 15 + 10, by vol.) followed by phenol + water + 5 N-ammonia (80 + 20 + 0·3, by wt.). Spots were detected by means of ninhydrin. The identity of sulphur-containing acids was checked by using samples of hydrolysates treated with hydrogen peroxide followed by chromatography as above. The presence of diaminopimelic acid in hydrolysates was confirmed by descending chromatography using as solvent a mixture of methanol + water + 10 N-hydrochloric acid + pyridine (32 + 7 + 1 + 4, by vol.; Rhuland, Work, Denman & Hoare, 1955).

Identification of amino sugars. Hydrolysates of cell walls were prepared as for the identification of amino acids and were examined by two-dimensional paper chromatography with solvents pyridine + water (4 + 1, by vol.) followed by *n*-butanol + acetic acid + water (6 + 1 + 2, by vol.; Salton & Pavlik, 1960), and also by one-dimensional chromatography with solvent pyridine + ethyl acetate + water + acetic acid (5 + 5 + 3 + 1, by vol.; Fischer & Nebel, 1955). Spots on replicate papers were detected by means of ninhydrin, alkaline silver nitrate and the Elson–Morgan reagents. Confirmatory evidence for the presence in hydrolysates of glucosamine and galactosamine was obtained by the method of Stoffyn & Jeanloz (1954). After partial separation by chromatography with *n*-butanol + ethanol + water (4 + 1 + 1, by vol.), the amino sugars were degraded by ninhydrin to the corresponding pentoses, which were separated by further chromatography with the above solvent and detected by aniline phthalate reagent.

Identification of sugars. Samples of cell walls were hydrolysed with 2 N-H_2SO_4 for 2 hr at 105°. After neutralization of acid by $Ba(OH)_2$ and removal of insoluble materials, the hydrolysates were examined by one-dimensional chromatography on Whatman no. 1 paper with the following solvents: the upper layer of ethyl acetate + pyridine + water (5 + 2 + 5, by vol.; Jermyn & Isherwood, 1949); isopropanol + water (4 + 1, by vol.); phenol + water (4 + 1, by wt.). Spots were detected by the aniline phthalate and silver nitrate reagents. The Dische reaction for the detection of aldoheptoses (Dische, 1953) was applied to samples of unhydrolysed cell walls following the method of Salton (1960). After 24 hr, the visible spectra of the solutions were obtained by using a Unicam SP. 500 spectrophotometer. In cases where the presence of aldoheptoses was indicated by this test, samples of hydrolysates were chromatographed and the Dische reaction was applied to solutions of the sugars eluted from chromatograms.

Extraction of lipids. Insufficient amounts of materials were available to permit a study of the effectiveness of different extraction procedures. The method adopted for the extraction of free and loosely bound lipids of the cell walls was that used by Few (1955). Samples of the cell walls were heated under reflux for 1 hr with 20 parts (v/w) of 95% (v/v) methanol in water to disrupt lipoprotein complexes. The methanol was removed under reduced pressure and the cell walls were dried (P_2O_5) for 3 hr *in vacuo*. The lipids were then extracted by heating under reflux with diethyl ether (5 ml.) for 30 min. Like all the solvents used, the ether was AnalaR grade, freshly dried and distilled. Three repetitions of the extraction removed all ether-soluble materials. The combined extracts were passed through a sintered-glass filter (no. 4 porosity) into a tared weighing bottle, the ether was removed by blowing a stream of nitrogen over the surface, and the residue weighed after drying (P_2O_5) overnight *in vacuo*. The lipids (pale yellow greases) were completely soluble in chloroform + methanol (2 + 1, by vol.), in which they were dissolved for storage at 4°.

For comparative purposes, the lipids from one batch of cell walls of *Pseudomonas aeruginosa* were extracted with chloroform + methanol (2 + 1, by vol.). The cell walls (180 mg.) were vigorously stirred with solvent (15 ml.) for 1 hr at room temperature. The insoluble residue was collected and washed on a sintered glass filter (no. 4 porosity), and the washings (30 ml.) were added to the filtrate. The extracted lipids were recovered as described above.

Analysis of lipids. Analytical studies were restricted by the small amounts (normally 10–15 mg.) of lipids from individual batches of cell walls. Estimations of phosphorus and nitrogen were made by the methods indicated above, and ester groups were determined by the method of Rapport & Alonzo (1955), with methyl palmitate (over 99% pure) as reference standard. A qualitative test for plasmalogens in lipid samples (about 1 mg.) was made by the method of Gray & Macfarlane (1958). Hydrolysates of lipids were prepared and examined by paper chromatography for the presence of amino acids, sugars and polyols (including inositol); papers run with *n*-butanol + diethyleneglycol + water (4 + 1 + 1, by vol.) were examined for choline by the phosphomolybdic acid method of Levine & Chargaff (1951). Samples (1–2 mg.) of the lipids were fractionated on silicic acid on the day following their extraction by the micro method of Lis, Tinocq & Okey (1961). The composition of whole lipids and lipid fractions was indicated by infrared spectroscopy.

Infrared spectra. Spectra were recorded on a Unicam SP. 100 double-beam spectrophotometer, with the specimens (cell walls or lipids) dispersed in discs of anhydrous potassium bromide.

Extraction of cell walls with EDTA. Samples of suspensions of cell walls (about 4 mg./ml.) in ion-depleted water were mixed with equal volumes of 0·0068 M-EDTA in 0·05 M-borate buffer (pH 9·2) or with borate buffer alone (Gray & Wilkinson, 1965). After 1 hr at 18–20°, the suspensions were centrifuged for 1 hr at 10,000 rev./min. and the supernatant fluids carefully withdrawn. The deposits of cell-wall residues were washed twice with ion-depleted water, freeze-dried, and their infrared spectra recorded. Samples of the supernatant fluids from the extractions were analysed for phosphorus and for carbohydrate (anthrone method). Estimates for the amounts of wall protein (containing aromatic amino acids) in the fluids were obtained by measurement of their absorptions at 275 mμ and comparing them with the values for whole wall suspensions. Corrections were applied for the absorption of EDTA when present.

RESULTS

Components of bacterial cell walls

Amino acids. As expected, acid hydrolysates of the cell walls from the four Gram-negative organisms studied contained a wide range of amino acids characteristic of proteins, as well as the mucopeptide component diaminopimelic acid. The range of amino acids and the relative amounts of each (assessed by visual examination of chromatograms) were similar for each organism. Alanine, glutamic acid and aspartic acid were present in the largest amounts, while the following acids were also identified: diaminopimelic acid, serine, glycine, threonine, lysine, arginine, tyrosine, phenylalanine, proline, valine, methionine, methionine sulphoxide, leucine/isoleucine, and cysteic acid (traces only). Hexosamines and ethanolamine were detected simultaneously. Allowing for the contributions by lipid and mucopeptide components to nitrogen analyses, the results in Table 1 indicate that the cell walls of these organisms contain about 40–50% of protein which resisted digestion by trypsin.

Amino sugars. The amino sugars identified in hydrolysates of the cell walls are shown in Table 2; values for total amino sugar contents are given in Table 1. In all

four cases, glucosamine was the most abundant amino sugar, while the greatest amount of galactosamine was in the walls from *Proteus mirabilis*. The low analyses for amino sugars are consistent with the low mucopeptide contents of the walls of Gram-negative organisms (Mandelstam, 1962).

Table 1. *Analyses of cell walls of some Gram-negative bacteria*

	Phosphorus (%)	Nitrogen (%)	Reducing sugar* (%)	Anthrone sugar* (%)	Amino sugar† (%)
Pseudomonas aeruginosa	2·3	7·7	18·1	6 7	3·9
Alcaligenes faecalis	2·3	8·5	15·2	3 8	3·7
Escherichia coli	1·5	9·5	19·1	4 4	4·3
Proteus mirabilis	0·95	8·8	20·8	4 0	3·3

* Expressed as glucose.
† Expressed as glucosamine (free base).

Table 2. *Sugar and amino sugar components of cell walls of some Gram-negative bacteria*

	Glucosamine	Galactosamine	Muramic acid	Glucose	Galactose	Rhamnose	Aldoheptose
Pseudomonas aeruginosa	+	+	+	+	−	+	−
Alcaligenes faecalis	+	+	+	+	−	+	?
Escherichia coli	+	−	+	+	+	−	?
Proteus mirabilis	+	+	+	+	+	−	+

Sugars. Table 2 indicates the neutral sugars which were identified as components of the cell walls. Except for aldoheptoses and the components of the walls of *Proteus mirabilis*, the sugars were readily identified chromatographically. In the case of *P. mirabilis*, a diffuse elongated spot covering the region corresponding to glucose and galactose was obtained. As aldoheptoses have similar R_F values, further information was sought by using the specific Dische reaction. The spectrum obtained from the unhydrolysed cell walls showed a pronounced absorption maximum at about 510 mμ, diagnostic for aldoheptoses. Similar spectra (Fig. 1) from reactions carried out on materials eluted from chromatograms confirmed this identification and also indicated the presence in the same region of the paper of glucose and galactose (absorption maxima at 410 and 390 mμ respectively). The application of the Dische reaction to the cell walls of the other organisms confirmed the chromatographic results and suggested the possible presence of aldoheptoses in the walls from *Alcaligenes faecalis* and *Escherichia coli*; however this was not satisfactorily confirmed by reactions with eluted materials.

Estimates of the reducing sugar and neutral sugar (anthrone method) contents of acid hydrolysates of the cell walls are given in Table 1. The values for reducing sugars (which include amino sugars) are likely to be overestimates (Salton & Pavlik, 1960), while those obtained by the anthrone method would be depressed by the low colour yields from galactose and rhamnose relative to that from glucose. Confirming the order of the anthrone results, inspection of paper chromatograms

suggested that the walls from *Pseudomonas aeruginosa* have the highest and those from *Alcaligenes faecalis* the lowest content of neutral sugars among the four organisms examined.

Lipids. As noted by Wardlaw (1963) and Herzberg & Green (1964), appreciable quantitative variation in lipid content and composition was found (Table 3) for different batches of cell walls from each organism. However, preliminary studies indicated that the lipids from all four organisms were broadly similar in composition. The analyses for lipid phosphorus indicated that lipids other than phospholipids were present in all cases. Fractionation of the lipids on silicic acid followed by infrared spectroscopic examination of the fractions showed that in every case only

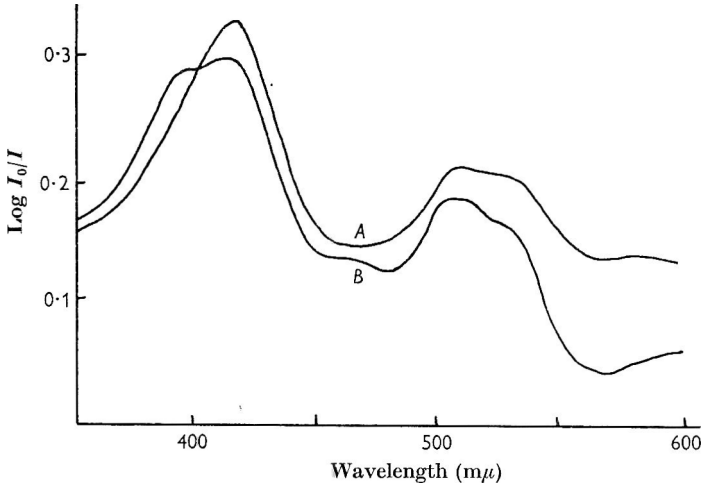

Fig. 1. Spectra of Dische reaction products of sugars from cell walls of *Proteus mirabilis*. The reaction was applied to fractions obtained by paper chromatography of hydrolysed walls (10 mg.). *A*, 'Glucose' fraction; *B*, 'galactose' fraction.

Table 3. *Readily extractable lipids of cell walls of some Gram-negative bacteria*

	Pseudomonas aeruginosa			*Alcaligenes faecalis*	
	Batch 1	Batch 2	Batch 3*	Batch 1	Batch 2
Lipid (%)	5·3	9·3	10·0	14·2	9·5
P in lipid (%)	2·1	2·5	2·2	0·73	1·2
N in lipid (%)	0·75	*c.*1	1·1	0·51	0·65
Ratio P:N	1·25	*c.*1·1	0·9	0·65	0·84
Ratio ester:P	2·0	1·9	2·0	2·1	2·0

	Escherichia coli		*Proteus mirabilis*	
	Batch 1	Batch 2	Batch 1	Batch 2
Lipid (%)	5·8	5·8	10·1	8·0
P in lipid (%)	1·87	3·14	0·48	1·5
N in lipid (%)	0·78	1·2	0·21	0·62
Ratio P:N	1·1	1·2	1·0	1·1
Ratio ester:P	1·9	1·8	2·2	1·7

* Lipids were extracted from this batch by chloroform+methanol (2+1, by vol.). A further 9·3% (expressed as % of the original walls) of fatty material was extracted by ether from an acid hydrolysate (Salton, 1953) of the residual wall material.

phospholipids and free fatty acids were present in large amounts. On the scale used, no detectable residues were obtained from the fractions which would have contained sterol esters and triglycerides, and neither sterols, nor monoglycerides nor diglycerides were detected in the fraction containing free fatty acids. The infrared spectra of the fatty acid fractions from *Pseudomonas aeruginosa* (Fig. 2) and

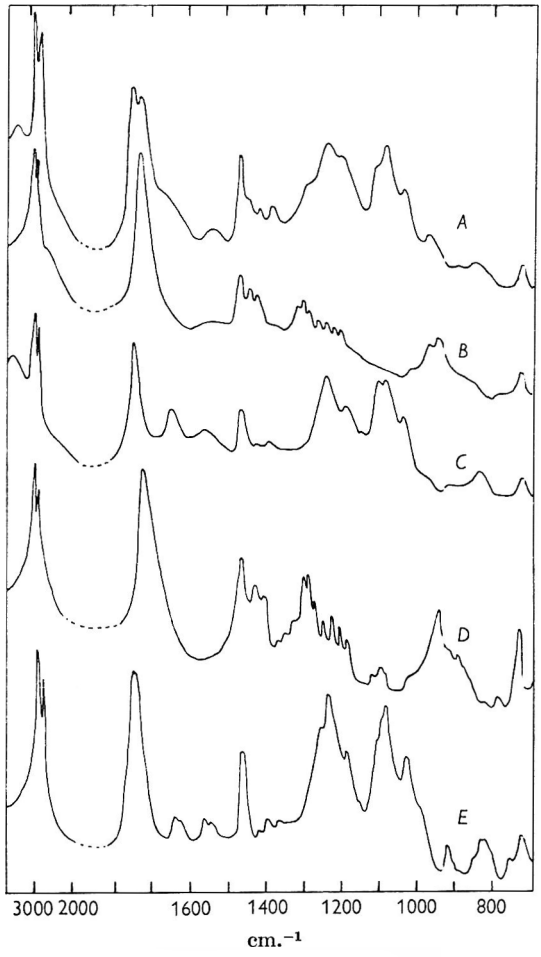

Fig. 2. Infrared spectra of lipids from cell walls of *Pseudomonas aeruginosa*. *A*, Unfractionated lipid; *B*, fatty acid fraction; *C*, phospholipid fraction; *D*, reference sample of palmitic acid; *E*, reference sample of L-α-(dipalmitoyl)phosphatidylethanolamine.

Alcaligenes faecalis showed absorption bands at about 970 cm.$^{-1}$, which may be attributed to *trans*-unsaturation in the component acids. This band was absent from the corresponding spectra of acids from *Escherichia coli* and *Proteus mirabilis*. Although decomposition of lipids, to give free fatty acids, during storage of the cell walls before extraction probably contributed to variability in lipid analyses, the presence of free fatty acids in the extracts cannot be entirely explained in this way, at least in the case of *P. aeruginosa*. Thus, about 50 % of the lipids from each of two batches of cell walls of *P. aeruginosa*, extracted one day after isolation by ether or by

chloroform + methanol, consisted of free fatty acids. No detectable amount of fatty acid was liberated from a pure sample of phosphatidylethanolamine on refluxing with 95% (v/v) methanol and then with ether as used in the standard extraction procedure. Thus the fatty acids appeared to be at least in part genuine components of the cell walls or were produced by autolytic reactions during the isolation of the walls.

The phospholipid fraction from the walls of each organism appeared to contain predominantly phosphatidylethanolamine, probably together with small amounts of phosphatidylserine. The infrared spectra of all the phospholipids resembled that of a synthetic sample of phosphatidylethanolamine (see Fig. 2 for that of the phospholipid fraction from *Pseudomonas aeruginosa*) and had an absorption band at 1030 cm.$^{-1}$, characteristic of this class of lipid (Marinetti & Stotz, 1954). The absence of a band at 970 cm.$^{-1}$ confirmed the absence of phosphatidylcholine. After acid hydrolysis of the phospholipids, choline, inositol and neutral sugars were not

Table 4. *Nitrogenous components of cell-wall lipids of some Gram-negative bacteria*

Pseudomonas aeruginosa	*Alcaligenes faecalis*	*Escherichia coli*	*Proteus mirabilis*
Ethanolamine*	Ethanolamine*	Ethanolamine*	Ethanolamine*
Serine*	Serine*	Serine	Serine
Glycine	Glycine		Hexosamine
Lysine	Lysine		
Alanine	Alanine		
Leucine	Leucine		
Hexosamine			

* Major components.

detected; ethanolamine was the major ninhydrin-positive component in all the hydrolysates. Whilst only traces of other amino compounds were found in the hydrolysate of lipid from *Escherichia coli*, significant amounts were found for those from the other organisms, particularly *Alcaligenes faecalis* (Table 4). Since glutamic and aspartic acids were detected in only small amounts and diaminopimelic acid was absent, it is unlikely that the lipid extracts were contaminated by insoluble wall material. The presence of proteo- or peptido-lipids in the phospholipid fractions from *P. aeruginosa* and *A. faecalis* would explain some of the differences (e.g. increased absorption at 1655 and 1550 cm.$^{-1}$, attributable to –CO.NH–) between the infrared spectra of these lipids and phosphatidylethanolamine. The origin of the component, provisionally identified as a hexosamine, in the lipid from *Proteus mirabilis* was not investigated.

A negative test for plasmalogens was obtained for all lipids, while the ester: phosphorus ratios of about 2:1 (Table 3) for the unfractionated lipids also indicate the essential absence of plasmalogens and lyso-phosphatides. The phosphorus: nitrogen ratios were near unity for the lipids from *Escherichia coli* and *Proteus mirabilis*, suggesting that nitrogen-free phospholipids such as cardiolipin and phosphatidylglycerol were unlikely to be major components. Although the situation with *Pseudomonas aeruginosa* and *Alcaligenes faecalis* was confused by the greater content of amino acids, further work on the lipids from *P. aeruginosa*, done in these laboratories by Mr J. W. Payne, has confirmed a similar view of these lipids.

Infrared spectra of cell walls

Qualitatively the spectra from different cell walls were very similar (Fig. 3), and showed the absorption bands expected for proteins, carbohydrates and lipids. The relative height of the major peaks at 1655 cm.$^{-1}$ and 1550 cm.$^{-1}$ was virtually constant (1·5 to 1), suggesting that these absorptions were almost solely due to peptide

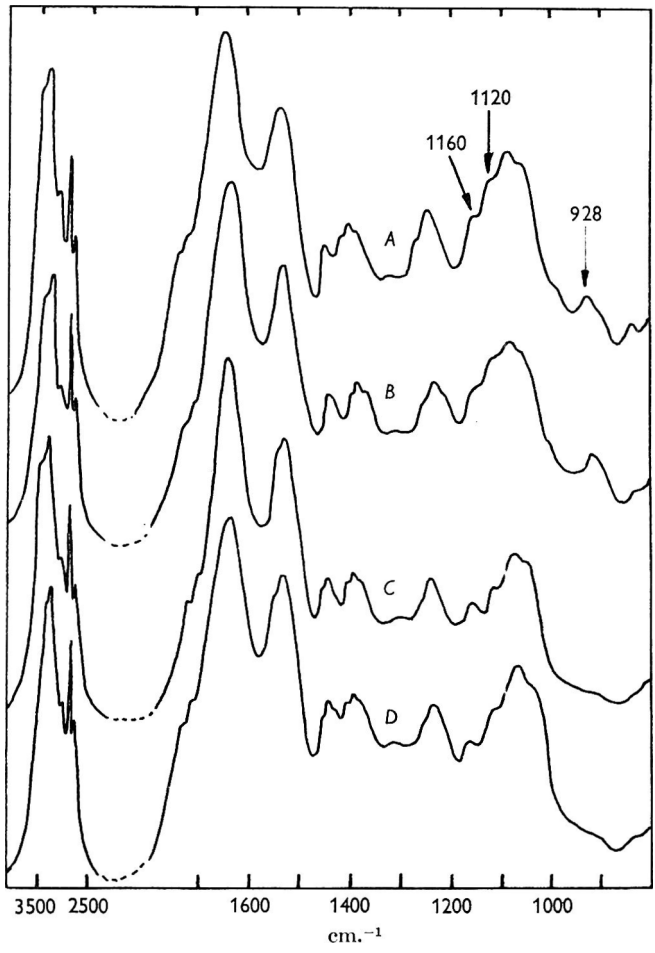

Fig. 3. Infrared spectra of bacterial cell walls. *A*, *Pseudomonas aeruginosa*; *B*, *Alcaligenes faecalis*; *C*, *Escherichia coli*; *D*, *Proteus mirabilis*.

bonds. By calibrating the 1655 cm.$^{-1}$ peaks by reference to a spectrum of trypsin, it was estimated that the cell walls contained 50–65% of material with peptide linkages (e.g. protein, mucopeptide).

The spectra of the walls from organisms which were highly sensitive to EDTA contained a minor peak at 928 cm.$^{-1}$ which was virtually absent from the other spectra. No firm assignment can be given to this absorption, although a band at 930 cm.$^{-1}$ has been attributed to a ring vibration in α(1–4)-linked polyglucosans (Stacey & Barker, 1960). Other differences between the wall spectra of EDTA-

sensitive and EDTA-resistant organisms were found in the region 1000–1300 cm.$^{-1}$, particularly the height of shoulders at 1120 cm.$^{-1}$ and 1160 cm.$^{-1}$. Although this region of the spectrum was too complex to permit detailed interpretation, phosphate esters and carbohydrates would be expected to make major contributions to absorption in this region.

Action of EDTA on cell walls

It has been shown (Gray & Wilkinson, 1965) that the turbidity of aqueous suspensions of the walls of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* is substantially decreased (about 30%) by 0·0034 M-EDTA at pH 9·2. After centrifuging such suspensions to deposit cell-wall residues, large proportions of the total phosphorus and carbohydrate, but not 'protein', remained in the supernatant fractions (Table 5). With both organisms, the percentage extractions of phosphorus and carbohydrate were similar, whereas the cell walls of *A. faecalis* contained appreciably less carbohydrate than those of *P. aeruginosa*. This suggested that EDTA solubilized most or all of the above components from a fraction of the cell walls, rather than the same fraction from the walls of both organisms.

Table 5. *Extraction by EDTA of cell-wall components of some Gram-negative bacteria*

Cell walls were treated with 0·0034 M-EDTA in borate buffer (pH 9·2) for 1 hr at 18–20°.

	Phosphorus	Carbohydrate	'Protein'	Decrease in turbidity† (%)
	Extraction (%)*			
Pseudomonas aeruginosa	30	35	1	33
Alcaligenes faecalis	28	29	4	28
Escherichia coli	2	3	2	14
Proteus mirabilis	0	0	0	0

* Corrected for extraction by buffer alone.
† See Gray & Wilkinson (1965).

In one experiment with the walls of *Pseudomonas aeruginosa*, only 7% of the soluble phosphorus was estimated as inorganic orthophosphate. It was also shown that the solubilization of components from the walls of this organism was not an artifact caused by incubating with enzymes in the purification of the walls (the phosphorus content of the walls was increased 15% by treatment with enzymes). However, the extractability of wall components by EDTA was decreased about 50% by omission of the enzyme treatment. In the control extractions with pH 9·2 buffer alone, rather high values for soluble phosphorus (19%) and carbohydrate (14%), compared with 'protein' (8%), were obtained with the walls of EDTA-sensitive organisms. Although the values could be decreased by more intensive centrifugation, the differences suggested solubilization by freeze-drying (Brown, 1958) or lability to alkali of specific wall components, perhaps of the same kind as solubilized by EDTA.

The selective solubilization of wall components by EDTA was confirmed by examination of the infrared spectra of the washed insoluble wall residues (Fig. 4). With the walls from *Pseudomonas aeruginosa* and *Alcaligenes faecalis*, EDTA

caused marked attenuation of the absorption bands in the range 1000–1300 cm.$^{-1}$ so that the spectra resembled much more closely those of the untreated cell walls of *Escherichia coli* and *Proteus mirabilis* (Fig. 3). Although EDTA caused some decrease in the turbidity of a suspension of the walls of *E. coli*, no significant extraction of wall components occurred with *E. coli* (the same batch of walls) or with *P. mirabilis*, and the infrared spectra were unaltered.

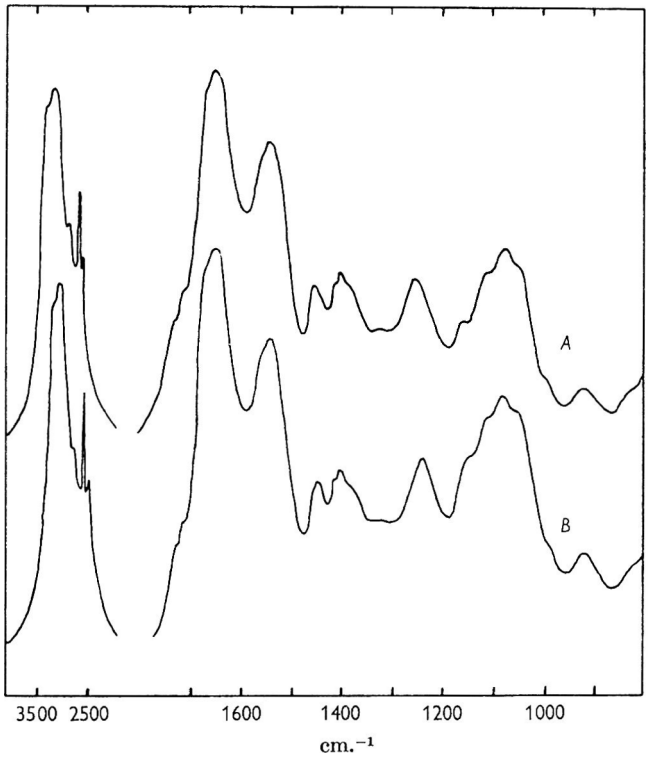

Fig. 4. The infrared spectrum of cell walls of *Pseudomonas aeruginosa* after treatment with EDTA. Cell walls were extracted for 1 hr at 18–20° with 0·0034 M-EDTA in borate buffer pH 9·2 (*A*) or with buffer alone (*B*).

DISCUSSION

The results of analyses on the cell walls from *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Escherichia coli* and *Proteus mirabilis* reflect the complexity of these structures in Gram-negative bacteria and resemble those obtained by other workers, although some differences (e.g. in monosaccharide components) were noted. The walls contained appreciable amounts of protein, polysaccharide and lipid, but relatively little mucopeptide.

Although detailed knowledge of bacterial lipids is increasing, little information is yet available about the nature of the lipids which are localized in the cell walls of Gram-negative bacteria. The lipids of the cell wall are of two types, of which one is released only after mild acid hydrolysis (Salton, 1953; Westphal & Lüderitz, 1954); only the readily extractable lipids are considered here. The lipids extracted from

whole cells frequently contain a high proportion of phospholipids, and of phosphatidylethanolamine in particular (Asselineau, 1962; Kaneshiro & Marr, 1962; Kanfer & Kennedy, 1963; Kates, Adams & Martin, 1964), while from limited studies the lipids of cell walls seem to be similarly constituted (Few, 1955; Cota-Robles, Marr & Nilson, 1958; Wylie & Johnson, 1962). Also peptido-lipids or lipid–amino acid complexes have been reported as components of ethanolamine-containing lipids from whole *Pseudomonas aeruginosa* (Silberman & Gaby, 1961; Sinha & Gaby, 1964) and *Alcaligenes faecalis* (Saito & Akashi, 1957). The present results are similar to those cited above and confirm the predominance of phosphatidylethanolamine in the phospholipid fractions. It has also been shown that substantial amounts of free fatty acids were present in the walls of the bacteria grown as described. Free fatty acids do not appear to have been previously identified in cell-wall lipids, but the neutral fraction of whole-cell lipid from *Escherichia coli* has been reported to consist largely of free fatty acid (Kaneshiro & Marr, 1962); similar reports have been made for other bacteria (O'Leary, 1962).

Although points of difference between the cell-wall lipids of organisms sensitive or resistant to EDTA have been noted, the most striking correlation between sensitivity to EDTA and composition of cell wall was found in the high phosphorus content and the similar monosaccharide components of the walls of sensitive organisms (Tables 1, 2). The significance of this observation was confirmed by the solubilization of phosphorus and carbohydrate components from cell walls of sensitive bacteria by EDTA. The cell walls of *Pseudomonas aeruginosa* and *Alcaligenes faecalis* appear to contain material not present or present only in small amounts firmly bound, in the walls of the more EDTA-resistant bacteria. Thus, the bactericidal activity of EDTA against the above organisms is probably to be explained by its action in solubilizing material essential to the integrity of their cell walls. Such an action is implicit in the claims of Campbell, Hogg & Strasdine (1962) and Norton, Bulmer & Sokatch (1963) to have prepared cell (protoplast) membranes of *P. aeruginosa* by using lysozyme+EDTA. By contrast, the cell walls of other Gram-negative organisms are not completely removed by treatment with lysozyme +EDTA (Holme, Malmborg & Cota-Robles, 1960; Murti, 1960; Salton, 1961).

Although the material extracted by EDTA in the present work has not yet been characterized, it seems likely to be lipopolysaccharide in nature. Thus, the extra phosphorus present in the walls of EDTA-sensitive organisms could not be accounted for by extractable phospholipid, and teichoic acids were not detected in wall preparations. The results of preliminary work done in these laboratories by Dr D. J. Byron and Mr J. W. Payne on material extracted by EDTA from the walls of *Pseudomonas aeruginosa* are strongly indicative of a lipopolysaccharide. Also, Homma, Suzuki & Ito (1963) showed that a water-soluble lipopolysaccharide was released by EDTA+lysozyme from the 'bacterial surface substance' (probably cell wall+membrane) of a strain of *P. aeruginosa*. The involvement of lipopolysaccharide rather than lipoprotein (Noller & Hartsell, 1961*a*, *b*) in the sensitization of Gram-negative bacteria to lysozyme by EDTA would be intelligible in view of the intimate association of lipopolysaccharide with mucopeptide (Weidel *et al.* 1960).

Although we assume that the action of EDTA involves metal cations present in the cell wall, it is not known whether the cations serve to bind lipopolysaccharide to

other components of the walls (see Humphrey & Vincent, 1962) or prevent release of lipopolysaccharide by inhibiting the action of autolytic enzymes which may be present in the walls. Calcium and magnesium have recently been detected in the lipid A component of lipopolysaccharides from *Escherichia coli* (Burton & Carter, 1964).

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A Model for Computer Identification of Micro-organisms

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SUMMARY

Specific groups (or taxa) of micro-organisms can be defined as to their location and extension in a space which is produced when the recorded characters are imagined as dimensions. Since a culture to be identified can be imagined as a point in this space, identification can be achieved by examining which group or taxon occupies the region where the 'unknown' lies.

INTRODUCTION

The use of electronic computers for identification purposes does not constitute a particular problem from the point of view of programming computers. However, as compared with the large literature on numerical classification procedures, the interest concerning computer identification has remained rather scanty. In a recent review by Sneath (1964) only a few references on this topic were given. Nevertheless, in connexion with identification, the requirement for a rapid treatment of available information is often much more pronounced than in connexion with classification. Where this need for obtaining results quickly is combined with the necessity of handling large quantities of material, computers may be successfully applied. An automatic identification procedure based on the principles of numerical classification and description of micro-organisms is presented below.

RESULTS

Background

The present author (Gyllenberg, 1965) has used a geometrical model for the description of microbial populations in numerical terms. The basic idea for this model was adopted from Silvestri and his co-workers (Dr L. R. Hill, private communication; Silvestri, Turri, Hill & Gilardi, 1962). According to Silvestri *et al.* bacterial cultures under study can be imagined as points in a multi-dimensional space, where the dimensions are given by the two-state tests used to characterize the cultures. By application of factor analysis to the primary data, the number of 'dimensions' can be decreased to a more manageable quantity, and, finally, classification can be based on the clusters or heaps of points (cultures) recognized in the imagined space.

In population description (Gyllenberg, 1965), the population can be defined in a given space by its centre of gravity and its extension (radius). The estimate of the centre of gravity is given by the means of the co-ordinates (e.g. x, y, z, respectively, in a three-dimensional space) of the cultures used to represent the population. As

an estimate for the radius, the empirical formula $r = 2\sqrt{(\Sigma d^2/n)}$ can be used. In this formula d is the distance from the points representing the individual cultures to the centre of gravity, and n is the number of cultures which make up the isolated sample of the population.

However, specific groups of cultures can be defined in a similar way. Automatic identification of an unknown culture is then possible by examining whether, when imagined as a point in a given space, it lies in the region occupied by one of the defined groups. If not, no one of the defined groups (i.e. defined taxa) shares the pattern of characters of the culture under examination; but, at any rate, the taxon to which it shows the greatest affinity can be recognized.

General procedure

Reference information. Identification along the above lines, like all identification procedures, requires a considerable amount of reference information. Accordingly, the reference information must first be collected according to the purpose of the identification procedure, processed and stored. The first step is to define a standard set of two-state tests for recording the corresponding characters on which the identification will be based. It lies outside the scope of this paper to discuss the principles for selecting the basic tests. It suffices to note that the particular identification purpose is of especial importance, but that yet other factors contribute to the information content of a given test.

The next step is to define the taxa or groups to be considered in identification and to select a suitable number of cultures as representatives of each taxon. These cultures can be referred to as *reference cultures*. The method of constructing or defining the reference taxa (i.e. whether monothetic or polythetic) does not directly influence the identification model (presuming that the taxa are really valid, which means that the representatives of each taxon show considerable mutual affinities). The characters of the reference cultures are then recorded by means of the selected series of basic two-state tests. The data thus obtained constitute the primary information, which defines the identification model.

Treatment of the primary information. The next step is to compute the correlation coefficients for each pair of characters from the primary reference information. To obtain significant coefficients, the total of reference cultures must be large: the number depends on the purpose of the identification and the kind and number of taxa to be considered, but a number greater than 100 can be given as a rough guide. Since two-state characters are used, the attribute A can be numerically expressed as 1, and the attribute not-A as 0, which makes it easy to obtain the correlation coefficients, even without computer aid. The correlation coefficients are arranged into a matrix, which provides the information needed for the principal component analysis.

The reference cultures when imagined as points are not evenly distributed in the multidimensional space which is defined by the basic characters (each character corresponding to a particular dimension), and accordingly their dispersion is hyper-ellipsoidal and not spherical. The *principal component analysis* (which is a particular kind of factor analysis; see Harman, 1960) determines the vectors or axes of the dispersion hyper-ellipsoid in decreasing order, namely, first the longest, then the

next longest orthogonal to the first, etc. To obtain a space with a manageable number of dimensions, the desired number of axes (or components) is selected in order of length. For population description, three dimensions have been considered sufficient (Gyllenberg, 1965). For identification purposes four or five may be needed, but for the sake of simplicity, only three-dimensional models are considered here. For the principal component analysis treatment by computer is required, but suitable routines may be available for most types of computer.

When the original multidimensional space has been reduced to a three-dimensional space, the final *identification space* is obtained. The next step, therefore, is to project the points which represent the reference cultures into the identification space. For this purpose a special computer routine is necessary. This 'projection routine' condenses the original information about each reference culture into three numbers (in the case of a three-dimensional identification space), namely, the three co-ordinates which indicate the location of the point in the identification space.

Definition of reference taxa in terms of identification space. Before the identification model can be used the reference taxa or groups to be considered in the identification procedure must be defined in terms of the identification space. For this purpose the centre of gravity and the radius of each taxon is computed according to the methods already described in the previous section (*Background*). Even with this model the borders between taxa which show clearly distinct centres of gravity may remain obscure, and some degree of overlap is perhaps impossible to avoid. However, as will be shown below, it is possible to overcome this confusion at least when the overlap is not too serious. At any rate, it is justifiable to examine the locations and extensions of the reference taxa in relation to each other. When considerable overlaps are found, e.g. when the centre of gravity of a given taxon is found to lie within the space occupied by another taxon, this may show that the reference material was inadequate and/or the set of basic tests was not properly selected, and that identification results may be of questionable value.

Identification procedure. For the 'unknown' organism to be identified the same characters have to be recorded as for the reference cultures. In this case too, this primary information is condensed according to the 'projection routine', and, in other words, the 'unknown' point can now be localized in the identification space. For this purpose the computer goes on to compare the 'unknown' with each one of the reference taxa. This is done by examining whether or not the distance from the 'unknown' point to the centre of gravity of the taxon considered is smaller than the corresponding radius. If not, the 'unknown' does not belong to this taxon or group. When the 'unknown' is found to belong to two groups (which is possible when the groups are mutually overlapping), the degrees of affinity of the 'unknown' to these groups can be evaluated from the relation $a = r/d$, where a stands for affinity, r is the radius of the group considered, and d is the distance from the 'unknown' to the corresponding centre of gravity. The organism to be identified can then be placed in the group to which it shows the greatest affinity. If the 'unknown' does not belong to one of the reference taxa, the affinity-figure can be used to indicate to which taxon or group it is most closely related.

For the identification procedure a special routine is needed. It is of course possible to store additional information concerning the reference taxa in the computer (e.g. the main pattern of characters), and to instruct the computer to indicate

on which points an organism identified as belonging to a certain taxon differs from the main character pattern. It may also be convenient to store even such information which has not been used to derive the identification premises (e.g. which pattern of sensitivity to antibiotics is likely to be found, expected heat and disinfectant tolerances, significance as an indicator of some specific source of contamination, etc.); the computer then provides these data together with the result of identification.

Table 1. *An illustration of the identification model*

The unit is derived from the basic assumption: positive character = 1, negative character = 0, which means that each character can be described as the segment of a line of length 1; x, y, and z are the co-ordinates corresponding to the three axes of the identification space; d is the distance from the culture (point) to be identified to the centre of gravity under examination; a is the affinity of the culture (point) to the corresponding reference group.

Isolate to be identified	Location in the identification space			Relation to the coliform group		Relation to the pseudomonas group	
	x	y	z	d_c	a_c	d_p	a_p
b06	3·53	0·51	0·26	0·28	1·21	1·32	0·56
b08	3·45	0·60	1·02	0·71	0·48	1·70	0·45
b20	3·36	1·68	−0·35	1·40	0·24	0·65	1·14
c15	3·06	1·00	−0·23	0·84	0·40	0·34	2·17
e45	3·25	0·43	0·12	0·54	0·63	1·00	0·74

An example of application. As a guide to the identification procedure presented above, the following simple example may be given. A model was prepared with the purpose of effecting identification of soil bacteria. Thirty-two simple tests were used to characterize the reference material which included representatives of several reference taxa. To recognize the most significant character correlations even the 'unknowns' to be identified (465 isolates in all) were used to derive the correlation coefficient matrix, and, accordingly, the identification space.

In this example coliform organisms and pseudomonads may be cited as reference groups. These groups were defined in terms of the identification space by the centre of gravity and the radius:

	Centre of gravity			Radius,
	x	y	z	r
Coliform organisms	3·61	0·76	0·35	0·34
Pseudomonads	3·02	1·15	−0·53	0·74

The distance between two points in a three-dimensional space is given by

$$d = \sqrt{[(x_1-x_2)^2+(y_1-y_2)^2+(z_1-z_2)^2]}.$$

When applied to the centres of gravity of the coliform organisms and the pseudomonads, respectively, this formula indicated a distance of 1·13, which showed that no overlap of these groups occurred, because $1{\cdot}13 > 0{\cdot}34+0{\cdot}74$.

For each 'unknown', the distances to the centres of gravity of the various reference groups were computed. For isolate b08 (see Table 1), the distance to the centre of gravity of the coliform group was

$$d_c = \sqrt{[(3{\cdot}61-3{\cdot}45)^2+(0{\cdot}76-0{\cdot}60)^2+(0{\cdot}35-1{\cdot}02)^2]} = 0{\cdot}71.$$

The affinity of isolate b08 to the coliform group was

$$a_c = 0{\cdot}34/0{\cdot}71 = 0{\cdot}48.$$

The 'identification data' for five 'unknowns' are given in Table 1. Of them isolate b06 obviously belongs to the coliform group because $a_c > 1$ ($r_c > d_c$). The isolates b20 and c15, again, belong to the pseudomonas group. Isolate b08 shows a rather low affinity to both the reference groups considered here, and may belong elsewhere, whereas isolate e45 shows a considerable affinity to both groups but, nevertheless, lies outside their spaces.

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Taxonomic Characteristics of So-called 'Form 2 Mycobacteria'

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SUMMARY

Two strains of rapidly growing spore-forming bacteria were obtained from cultures of *Mycobacterium tuberculosis* and identified as so-called 'form 2 mycobacteria' (Csillag, 1961). The properties of these and of two similar strains received from Dr A. Csillag were investigated, and compared with those of NCTC strains of the genus *Bacillus*. All four 'form 2' strains were almost identical in character, and differed from *M. tuberculosis* in many respects, rendering it highly unlikely that they were genetically derived from the mycobacterial cultures in which they were found. They were identified as strains of *Bacillus licheniformis*. It is considered that 'form 2' organisms are not part of a complex mycobacterial life-cycle, as has been suggested, but are contaminants.

INTRODUCTION

The repeated isolation of rapidly growing bacteria from cultures of tubercle bacilli and 'anonymous' mycobacteria was reported by Csillag (1961). The chief characteristics of this observation were as follows. The mycobacterial cultures had to be incubated with aeration for 2–3 months for the growth to appear, and this growth consisted of rods which were not acid-fast, which stained variably by Gram's method and possessed endospores. Once manifest, these organisms grew readily on ordinary nutrient agar within a day or two. It was clear that the properties of these organisms differed greatly from those of the mycobacterial cultures in which they were found. On the other hand, they had some characteristics of bacteria of the genus *Bacillus*. Contamination was considered by Csillag as an explanation of the findings, but it was held that the precautions taken against it, the failure of the growths to appear in the controls, and the properties of the growths themselves rendered this explanation very unlikely. Csillag therefore concluded that they were actually derived from the mycobacteria present and represented an alternative form of existence of these organisms. For convenience of description she gave the name 'form 2' to the non-acid-fast growths, 'form 1' denoting the acid-fast organisms in this terminology. Further accounts of the emergence of form 2 strains in mycobacterial cultures growing on autoclaved Löwenstein–Jensen medium, and of their properties, have been published more recently (Csillag, 1962, 1963*a*, *b*, 1964*a*, *b*).

In view of the fundamental importance of these conclusions I tried to confirm the emergence of form 2 strains under the conditions described, and to assess their properties and significance. While it has not been possible to achieve consistent

isolation of such organisms, a few have been obtained. It is the purpose of this paper to describe the isolation of two of them and to outline their characteristics and those of two of Dr Csillag's own strains.

METHODS

Bacterial strains

Mycobacteria. Stock cultures: *Mycobacterium tuberculosis* H37Rv (×4) (see below for the meaning of (×4), etc.) and H37Ra (×2); *M. bovis* NCTC 9320, and a BCG strain obtained from Messrs Glaxo Ltd., Greenford, Middlesex (×4); *M. microti* ('vole bacillus') NCTC 8710 (×2); *M. kansasi* ('photochromogens'), 8 strains, nos. C12 (×2), 120, 335 (×2), 1640, 4619 (×2), 4777 (×2), 8841 (×2), 'Price' (×2) (these were obtained from Dr W. Pagel, Clare Hall Hospital, Barnet, with the exception of strain 'Price', obtained from Dr R. W. Riddell, Brompton Hospital, London). In the experiment described below for the isolation of form 2 bacteria, one culture of each strain was used, except as indicated above by the figure in parentheses, e.g. 'H37Rv (×4)', which indicates that four cultures of the H37Rv strain were used in that experiment.

Recent isolates of Mycobacterium tuberculosis. These consisted of 29 cultures from 25 strains, 4 of the strains being used in duplicate. These strains had been collected from the clinical laboratory over several months and were from the sputa of 25 untreated cases of pulmonary tuberculosis; all were isoniazid-sensitive and were nicotinic acid-positive by the method of Gutierrez-Vazquez (1958). Thirteen of the strains were the primary isolates themselves which had been incubated for 4–10 weeks from first appearance of growth, and stored at room temperature for periods between 1 and 16 weeks. The remainder were first or second subcultures of primary isolates.

Bacillus species. B. licheniformis, 3 strains (NCTC 6346, 7589, 8233); *B. subtilis*, 2 strains (NCTC 3610, 8236); *B. pumilus*, 2 strains (NCTC 7576, 8241); *B. cereus*, 1 strain (NCTC 7587).

Form-2 organisms. For convenience and clarity, the different form 2 strains referred to in this paper are designated with the prefix 'FT'. Two strains were received on agar slopes from Dr A. Csillag: one of them, strain FT1, had been found in a culture of *Mycobacterium tuberculosis*, no. I1413, from a British patient, and strain FT2 was from a similar culture, no. 59416, from an Indian patient.

General cultural methods

Mycobacterial cultures. Primary isolations of the strains of *Mycobacterium tuberculosis* from sputum were made by the trisodium phosphate method (Peizer, Chaves & Widelock, 1954), the treated deposits being grown on Löwenstein–Jensen medium (*Mackie and McCartney's Handbook*, 1960) without potato starch (Jensen, 1955) dispensed in 3 ml. amounts in ½ oz. screw-capped bottles. Batches of the same medium without malachite green were also used. Strains of mycobacteria were maintained on Löwenstein–Jensen medium and were subcultured at intervals of a few months.

Bacillus species and form 2 cultures. The dried cultures received from the National Collection of Type Cultures were reconstituted with broth, and a loopful plated on nutrient agar. After incubation for 2 days a single representative colony was sub-

cultured on a nutrient agar slope (the primary culture), which was incubated for 3 days at 37° with the screw cap loose to encourage sporing, and then kept at room temperature indefinitely with the cap screwed tight. A single subculture from each primary culture was made on a nutrient agar slope treated similarly (secondary culture) and all the subcultures needed for the present work were made from the secondary cultures; the primary cultures were held in reserve in case of possible contamination of the secondary cultures. The nutrient agar slopes of the two form 2 strains (FT 1, FT 2) received from Dr Csillag were treated as primary cultures. After subcultivation of the growth from the slope and the condensation water to nutrient agar plates to test for purity, a secondary culture was made from each slope and used for all further tests. The two strains of form 2 bacteria (FT 3, FT 4) isolated during this work were subcultured to nutrient agar plates to test purity, and a single colony from each used to obtain primary and secondary cultures on nutrient agar slopes as described. Nutrient agar plates used for testing purity were incubated for 1 week.

The nutrient broth used was made from meat extract and peptone (Oxoid no. 2, Oxo Ltd., London), dispensed in 10 ml. amounts in $\frac{1}{2}$ oz. screw-capped bottles; nutrient agar consisted of the same medium solidified with 1·5% (w/v) agar, dispensed in slopes in similar bottles, or in plates. Blood agar was made by adding about 7% (v/v) oxalated horse blood (Wellcome Laboratories, Beckenham, Kent) to nutrient agar, previously melted and cooled to 50°. Blood agar plates were of the usual 'sandwich' pattern (*Mackie and McCartney's Handbook*, 1960). Except as stated below, media were sterilized by autoclaving at 120° for 15 min. Incubation was aerobic at 37°, except where otherwise indicated.

Special differential tests

Special differential tests were used for the purpose of discovering similarities or otherwise between the four form 2 strains and the three species of *Bacillus* on the one hand, and the species *Mycobacterium tuberculosis* on the other. Many of the tests used were identical with or based on those described by Knight & Proom (1950) in a paper on the physiological characteristics of the *Bacillus* genus. Except where otherwise specified, the media used in these tests were inoculated from the condensation water of the stock slopes of the four strains of form 2 organisms, the three NCTC strains of *B. licheniformis*, and, where necessary for comparison, the other *Bacillus* strains. The minimum amount of condensation water held by a 3 mm. platinum loop was used. Any culture which showed growth was subcultured on a nutrient agar plate, incubated for 3 days and identified by its colonial and microscopic morphology. In a few tests inocula of mycobacteria were used: surface growth from a 4-week-old Löwenstein–Jensen slope was homogenized in sterile distilled water with the aid of glass beads as described by Csillag (1962), and a loopful of the homogenate used as inoculum.

Growth at different temperatures. Cultures were made in nutrient broth and incubated at 45°, 37° and room temperature (18–20°). A thermostatically controlled water bath was used for incubation at 45°, the cultures being totally immersed in it. The cultures were examined daily up to 3 days, and the amount of growth (turbidity and pellicle formation) recorded.

Motility. Overnight broth cultures at 37° were examined microscopically by a hanging-drop preparation.

Anaerobiosis. Nutrient agar plates were inoculated in duplicate, and one plate of each pair incubated up to 3 days anaerobically in a McIntosh and Fildes jar. The other plate was incubated aerobically for comparison.

Growth in the presence of 4% sodium chloride. Parallel cultures were made in nutrient broth with and without 4% NaCl (w/v) and incubated for 2 days. In addition, Dubos–Davis fluid medium (*Mackie and McCartney's Handbook*, 1960) was made up with and without 4% NaCl (w/v) and distributed in 10 ml. amounts in $\frac{1}{2}$ oz. screw-capped bottles. These were inoculated with the four FT strains and the three *Bacillus licheniformis* strains, and with 6 strains of *Mycobacterium tuberculosis* (the H37Rv strain and 5 strains recently isolated from sputum). The mycobacterial cultures were incubated for 28 days (when all the control cultures without added NaCl showed good growth) and the remainder for 2 days.

Voges–Proskauer reaction, nitrate reduction and citrate utilization. Cultures in M.R.V.P. medium (Oxoid) were incubated for 2 days and tested for the formation of acetylmethylcarbinol (*Mackie and McCartney's Handbook*, 1960). Reduction of nitrate to nitrite was tested by the method of Skerman (1959): samples were taken after 1, 2 and 3 days of incubation and tested for nitrite formation by a spot test on a white tile with starch + iodide indicator. Cultures were also tested for the production of gas from nitrate under anaerobic conditions (Gibson, 1944). Citrate utilization was tested by inoculating Koser's citrate medium (Oxoid), incubating for 2 days, and observing the development of turbidity; a loopful of any growth thus seen was subcultured to a new bottle of the same medium, and citrate utilization was assumed to occur only when the second subculture also showed growth of the strain under test. In these tests, the NCTC strains of *Bacillus subtilis* and *B. pumilus* were also used.

Action on carbohydrates. The ammonia basal medium of Knight & Proom (1950) was used: this medium contains ammonium phosphate as the only source of nitrogen, and whichever carbohydrate is added constitutes the only source of carbon. It was found convenient to adopt the following modifications: the medium was adjusted to pH 7·4 and phenol red 0·01% (w/v) and agar 1·5% (w/v) were added. Stock solutions of the carbohydrates, 10% (w/v) in distilled water, were sterilized by Seitz filtration and were added to the melted basal medium to a final concentration of 1% (w/v). The medium was dispensed as slopes in $\frac{1}{2}$ oz. screw-capped bottles. The bottles were incubated for 3 days; surface growth indicated utilization of the carbohydrate tested, and yellow coloration of the medium indicated the development of an acid reaction. In addition to the form 2 strains and *Bacillus licheniformis* strains, the *B. subtilis*, *B. pumilus* and *B. cereus* strains were also tested in order to confirm that the results obtained with the medium were similar to those described by Knight & Proom (1950). None of the organisms grew on the basal medium without the addition of carbohydrate. The formation of CO_2 gas from glucose under semi-anaerobic conditions was also tested for. The test was done as described by Gibson & Abd-el-Malek (1945) with the following modifications. The medium consisted of 5% (w/v) peptone (Oxoid) with 5% (w/v) glucose in distilled water, adjusted to pH 7·3, and distributed into U-tubes each having one closed limb which was completely filled with medium. The surface of the medium in the open limb was sealed after inoculation with a layer of paraffin wax 1 cm. deep. After incubation up to 5 days, the amount of bacterial growth and the size of any gas bubbles in the

closed limb were noted; shrinkage of the latter after the addition of alkali indicated their CO_2 content. *Bacillus subtilis* and *B. pumilus* strains were included in this part of the investigation.

Enzymic activities. (1) *Urease.* The rapid test for urease activity of Elek (1948) was used. The form 2 and NCTC culture of *Bacillus* species were grown for 2 days on nutrient agar, and a loopful of the growth used as the inoculum. The same 6 cultures of *Mycobacterium tuberculosis* referred to earlier in this section were also tested for urease production by this technique: a well-loaded loopful of the surface growth formed the inoculum.

(2) *Hydrolysis of starch, gelatin and egg albumen.* For starch hydrolysis a 5% (w/v) solution of soluble starch (AnalaR) in distilledwater was sterilized by steaming for 1 hr on three successive days. Five drops of this solution were added with a Pasteur pipette to 10 ml. nutrient broth, which was then inoculated. After incubation for 2 days a few drops of the culture were placed on a white tile and Gram's iodine solution added: failure to develop a blue-black coloration indicated complete hydrolysis of the starch. In cases of doubt, the culture was re-incubated for 2 days after the addition of 5 more drops of starch solution and re-tested. Negative controls were provided by the use of uninoculated starch broth incubated similarly, and of cultures inoculated with *Bacillus pumilus* (Knight & Proom, 1950). For gelatin hydrolysis bottles ($\frac{1}{2}$ oz.) containing 10 ml. nutrient broth solidified with 10% gelatin were inoculated and incubated at 37° up to 5 days. The cultures were tested daily by bringing them to 0–4° in a melting ice bath. Failure to solidify at this temperature indicated gelatinase action. Control uninoculated bottles of gelatin medium were tested in parallel. Egg albumen hydrolysis: liquefaction of Löwenstein–Jensen medium by form 2 cultures was confirmed, and compared with the three type strains of *B. licheniformis*, by inoculation on to slopes of this medium made up without malachite green, with incubation up to one week.

Sensitivity to chemotherapeutic agents. Isoniazid sensitivity. Dubos–Davis fluid medium (see above) containing isonicotinyl hydrazide 500 μg./ml. was used. Screw-capped $\frac{1}{2}$ oz. bottles containing 10 ml. of this medium were inoculated and incubated for 2 days. *Other drugs.* Sensitivity to penicillin, streptomycin, tetracycline, chloramphenicol, erythromycin and sulphadimidine was tested by the disc diffusion plate technique of Fairbrother & Martyn (1951) on the medium of Jewell & Pearmain (1954). The amount of each drug incorporated in the discs was such as to produce inhibition zones of 15–20 mm. diameter after overnight incubation on plates inoculated with the standard sensitive Oxford strain of *Staphylococcus aureus.*

Endospore characteristics. (1) *Preparation of spore suspensions.* The four strains of form 2 organisms and three *Bacillus licheniformis* strains were inoculated into $\frac{1}{2}$ oz. bottles of nutrient broth and incubated for 4 days with the caps of the bottles loose. The bottles were shaken vigorously once each day (with the cap tight) to break up the pellicles and to increase aeration. The contents of each bottle were homogenized in a sterile Griffith hand-operated glass grinder, and the bottles allowed to stand for 10 min. so that any coarse particles remaining could settle. The turbid supernatant fluid was separated and 3 ml. volumes stored in $\frac{1}{4}$ oz. screw-capped bottles at 4°. Microscopic examination of stained smears at this stage showed that the great majority of the organisms were in the spore form.

(2) *Heat resistance.* From each suspension before heating a standard loopful was taken and spread evenly over the surface of a nutrient agar plate. Samples (2 ml.) of each suspension were placed in $\frac{1}{4}$ oz. bijou bottles, which were totally immersed in a water bath already at the required temperature. The bottles were shaken gently during the first 5 min. of each period of heating to ensure the even distribution of heat through the suspension. After the chosen period of time had elapsed, a loopful of the heated suspension was spread on a plate of nutrient agar as in the case of the unheated control. All the plates were incubated overnight and the amounts of growth on each compared. The following temperature/time exposures were used: 70°/30 min., 80°/30 min., 90°/20 min., 100°/20 min.

Inhibition by malachite green in Löwenstein–Jensen medium. Several techniques were used to test the capacity of form 2 organisms and *Bacillus* strains to grow on Löwenstein–Jensen medium with and without malachite green; these will be described elsewhere. In general, assessments were made of the power of vegetative forms, unheated spores and spores heated at 80°/30 min. to initiate growth from inocula varying in size from about 10 up to 10^6 viable organisms on Löwenstein–Jensen medium, with and without malachite green, dispensed in bottles or in plates.

Special differential tests. Smears were allowed to dry at room temperature and were heat-fixed. Gram, Ziehl–Neelsen and spore staining methods were done as described by Csillag (1961), except that spirit (90 %, v/v, ethanol in water) was used instead of acetone for decolorization in Gram's method.

RESULTS AND DISCUSSION

The isolation of form 2 bacteria

Fifty-six cultures of mycobacteria on Löwenstein–Jensen medium were used. They comprised 27 recent isolates of *Mycobacterium tuberculosis*, together with 27 stock cultures, 13 of these being *M. kansasi*. The origin and previous handling of these cultures is described above; at the time the experiment was started each culture bottle contained a well-developed surface growth. Initially, each culture was checked to ensure that the growth did consist of acid-fast bacilli, and it was then subcultured to nutrient agar to make sure that no form 2 or other organisms were already present. The cultures were then incubated for 20 weeks; they were aerated intermittently during this period by removing the screw cap of the bottle for a few seconds twice a week. Once every 2 weeks the surface growths were subcultured to nutrient agar plates to detect form 2 bacteria. From this experiment only two form 2 strains were obtained: one appeared at the 6th week of incubation with aeration, and the second at the 8th week. Both appeared in bottles containing primary isolates of *M. tuberculosis* obtained from the sputa of different patients, and their presence in the cultures was obvious even before subculture because the egg medium turned brown and became liquefied. Each gave a confluent growth on nutrient agar after overnight incubation. None of the remaining 54 cultures yielded growth on nutrient agar before being discarded after a total of at least 24 weeks' incubation (4–10 weeks preliminary, 20 weeks with aeration). Stock cultures were made and the two form 2 strains thus isolated were designated FT 3 and FT 4. Subcultures were sent to Dr A. Csillag: she confirmed that they were indeed form 2 organisms, and kindly sent me two of her strains for comparison. The characteristics

of these four form 2 strains were then investigated; since early findings suggested a strong resemblance to organisms of the genus *Bacillus*, NCTC strains of this genus (enumerated above) were tested in parallel.

The properties of form 2 bacteria

Morphology and staining reactions. All four strains consisted of rods which were fairly consistent in thickness (about 0·8μ) but variable in length. Overnight cultures in broth or on the surface of nutrient agar showed mainly rods 5–7μ in length. Long filaments were also seen; these on close inspection were streptobacillary in form, consisting of closely connected rods of the length indicated above.

Overnight cultures usually showed occasional spore-containing bacilli; at 2 days nearly all showed spores; and at later times the cultures consisted almost entirely of spores lying free or arranged in chains, with only a few bacillary bodies remaining. The appearances presented by spore-containing form 2 bacteria were those of Bacillus species of morphological group 1 (Smith, Gordon & Clark, 1946), in which the sporangium is only slightly swollen, or not swollen at all, with a thin-walled oval spore.

The growths were invariably not acid-fast. They were easily stained at room temperature by methylene blue or other simple stains, and under suitable conditions were Gram-positive. This was a variable and unpredictable property, however, and more often than not the organisms appeared to be Gram-negative. At times different rods in the same chain would show a different Gram-staining reaction. These morphological and staining properties of the form 2 strains, however, resembled closely those of the NCTC strains of *Bacillus licheniformis*, when the latter were grown under similar conditions and stained on the same slides.

Cultural characteristics. The aerobic growth of these form 2 strains in nutrient broth and on nutrient agar was very rapid. After overnight incubation at 37° in broth, a firm surface pellicle was formed, with moderate general turbidity. On microscopic examination in fresh preparation, all four strains showed very active motility. On nutrient agar large colonies (3–4 mm. diameter) were formed after incubation for 24 hr. The appearance of these colonies was extremely variable, and very dependent on the type, or even batch, of medium used, as well as on other unpredictable factors in the cultural conditions. In general, early colonies were grey, semi-translucent and faintly greenish in hue, and rhizoid in character. Later, particularly on media containing blood or serum, small patches of mucoid growth appeared, resembling beads of slightly milky fluid resting on the rhizoid growth. At times the growth was entirely mucoid, but usually the mixed mucoid + rhizoid appearance was seen; this is well illustrated in fig. 5, plate 2, of Dr Csillag's paper (Csillag, 1961). Growths on protein-containing medium, especially on Löwenstein–Jensen medium without malachite green, became pigmented after a few days of incubation, showing a strong reddish purple colour. Surface growths from heavy inocula on blood agar, taken freshly from the incubator, had the strong, sour, musty and offensive smell characteristic of some species of the genus *Bacillus*, and wide zones of haemolysis, with greenish staining, were formed round the colonies. No diffusible pigment was formed in the absence of blood. In all these general cultural characteristics, the form 2 organisms were indistinguishable from the NCTC strains

of *Bacillus licheniformis*, and were quite unlike mycobacteria in general, and *Mycobacterium tuberculosis* in particular.

The results of the specific differential tests used in investigating these organisms, together with some of those already mentioned, are set out in Table 1. Those tests marked with an asterisk were used by Knight & Proom (1950) for characterizing and distinguishing various species of the genus *Bacillus*. Most of the properties of the form 2 strains were re-tested after repeated single-colony subcultures, and also after the stock cultures had been kept for several months, and were found to be unaltered.

Table 1. *A summary of the characteristics of four form 2 strains (FT1 to 4) and a comparison of their properties with those of three strains of Bacillus licheniformis (NCTC 6346, 7589, 8233) and with those of Mycobacterium tuberculosis*

	NCTC 6346	NCTC 7589	NCTC 8233	FT 1	FT 2	FT 3	FT 4	*M. tuberculosis*
Growth in 24 hr in broth at 37°	+	+	+	+	+	+	+	−
*Growth at 18°	+	+	+	+	+	+	+	−
*Growth at 45°	+	+	+	+	+	+	+	−
*Growth in 4% NaCl medium	+	+	+	+	+	+	+	−
*Growth under anaerobiosis	+	+	+	+	+	+	+	−
*Growth in synthetic medium with glucose	+	+	+	+	+	+	+	−
*Citrate utilisation	+	+	+	+	+	+	+	−
Colonies 3–4 mm. diam. formed in 24 hr	+	+	+	+	+	+	+	−
Haemolysis on blood agar	+	+	+	+	+	+	+	−
Purple-red pigment formation	+	+	+	+	+	+	+	−
Surface pellicle in 24 hr	+	+	+	+	+	+	+	−
Acid-fast	−	−	−	−	−	−	−	+
*Rods 0·8 μ diam.	+	+	+	+	+	+	+	−
Variable Gram reaction	+	+	+	+	+	+	+	−
*Motile	+	+	+	+	+	+	+	−
*Endospore formation	+	+	+	+	+	+	+	−
*Nitrate reduction	+	+	+	+	+	+	+	+
*Voges-Proskauer reaction	+	+	+	+	+	+	+	N
*Acid from xylose	+	+	+	+	+	+	+	−
*Acid from arabinose	+	+	+	+	+	+	+	−
Acid from mannitol	+	+	+	+	+	+	+	−
*Gibson-Abd-el-Malek reaction	+	+	+	+	+	+	+	−
*Urease	−	−	−	−	−	−	−	+
*Starch hydrolysis	+	+	+	+	+	+	+	N
*Gelatine liquefaction	+	+	+	+	+	+	+	−
Egg albumen liquefaction	+	+	+	+	+	+	+	−
Sensitive to isoniazid	−	−	−	−	−	−	−	+
Sensitive to penicillin	−	−	+	+	−	+	+	−
Sensitive to streptomycin	+	+	N	+	+	+	+	+
Sensitive to tetracycline	+	+	N	+	+	+	+	+
Sensitive to chloramphenicol	+	+	N	+	+	+	+	+
Sensitive to erythromycin	+	+	N	+	+	−	+	−
Sensitive to sulphadimidine	+	+	N	+	+	+	+	+
Sensitive to malachite green	+	+	+	+	+	+	+	−

* Differential characteristics of *B. licheniformis* (Knight & Proom, 1950).

+, Indicates possession of the property described; −, indicates non-possession of the property described; N, indicates strain not tested, or no reference in literature.

Where valid comparison is possible, the characteristics of *Mycobacterium tuberculosis* are also included in Table 1. These have been taken from *Topley & Wilson's Principles* (1964) and Soltys, St Hill & Ansell (1952), supplemented by reference to other authors cited in the text below, or by experimental work forming part of the present work. Several of the results recorded in Table 1 need no discussion at this stage; others need further comment and are discussed below.

Effect of cultural conditions. The form 2 strains are clearly able to grow in a wide variety of nutritional environments and of conditions of incubation, in which they differ from *Mycobacterium tuberculosis*. Of particular note is their power to grow under strictly anaerobic conditions, in which their growth was only slightly less than when grown aerobically. This is in very marked contrast to *M. tuberculosis*, which is killed by anaerobic incubation (Loebel, Shorr & Richardson, 1933; Guy, Raffel & Clifton, 1954). No literature reference was found to the effect of 4% NaCl on mycobacteria. Six strains of *M. tuberculosis* were therefore tested in fluid medium containing 4% NaCl; all six strains did not grow. The form 2 and *Bacillus licheniformis* strains showed equally good growth in this medium and in nutrient broth whether with added NaCl or not. This property was found by Knight & Proom (1950) to be possessed by almost every species in the genus *Bacillus* which they tested.

Biochemical and enzymic activities. Formation of nitrite from nitrate occurred with the form 2 strains, and with *Bacillus licheniformis* and *B. subtilis* but not with *B. pumilus* in agreement with Knight & Proom (1950). This was one of the few characters which form 2 strains had in common with mycobacteria (Virtanen, 1960; Hedgecock & Costello, 1962); however, the formation of nitrogen gas from nitrate (Gibson, 1944) and of carbon dioxide from 5% glucose (Gibson & Abd-el-Malek, 1945), which was found with both form 2 and *B. licheniformis* strains, cannot occur with the mycobacteria because of their requirement of aerobic growth.

Utilization of citrate as sole carbon source. This was found with all four form 2 strains. Although citrate enhances the growth of *Mycobacterium tuberculosis*, it is apparently not utilized as sole carbon source (Edson, 1951). The form 2 strains also grew well, with acid production, from small inocula on the defined medium with ammonia as the sole source of nitrogen, and glucose, xylose, arabinose or mannitol as carbon source. The action of *Bacillus licheniformis* on the first three of these carbohydrates was described by Knight & Proom (1950) and on mannitol by Cowan & Steel (1961). Although large inocula of certain strains of *M. tuberculosis* can initiate growth on similar defined media, small inocula cannot do so without the addition of more complex organic materials, especially in the case of recently isolated strains (Middlebrook & Dubos, 1958). Although glycerol and glucose can be assimilated by *M. tuberculosis*, xylose, arabinose and mannitol cannot (Edson, 1951; *Topley & Wilson's Principles*, 1964).

Urease formation. Urease is formed by mycobacteria with the exception of *Mycobacterium avium* and certain related strains (Pawlicki, Hertz & Green, 1963; Oka & Yamaguchi, 1963), and all 6 strains of *M. tuberculosis* tested by the method of Elek (1948) were positive; the form 2 strains and *Bacillus* strains were negative. Knight & Proom (1950) also found these *Bacillus* species to be urease-negative.

Hydrolysis of starch, gelatin and more complex proteins. These properties are characteristic of *Bacillus licheniformis* (Knight & Proom, 1950) and were found to be possessed by the form 2 strains. It is common knowledge that the mycobacteria do

not digest Löwenstein–Jensen medium, or other coagulated egg media, nor do they liquefy gelatine (Skerman, 1959). There is no information about the action of *Mycobacterium tuberculosis* on starch in the literature references examined and this property was not investigated.

The effect of antibacterial agents. The form 2 strains differed from *Mycobacterium tuberculosis* in being highly resistant to isoniazid, and, in the case of three of them, in being penicillin-sensitive. In the cases of the other five drugs tested, the form 2 strains did not differ significantly from the *Bacillus licheniformis* strains in their sensitivity patterns.

The reasons for testing the capacity of form 2 strains to grow on Löwenstein–Jensen medium with and without malachite green, and the detailed results, will be described elsewhere. In general, the spores of the form 2 strains did not initiate growth on this medium containing malachite green; the vegetative forms did so in a rather capricious way, with the exception of strain FT 1, which did not yield growth after incubation for 12 days from inocula ranging from 10 to 10^4 viable organisms. However, small inocula of the spores or vegetative forms of all four strains grew as readily on Löwenstein–Jensen medium without malachite green as on blood agar. The *Bacillus licheniformis* cultures, tested in parallel, showed very similar patterns of inhibition by malachite green. These characteristics contrast with those of *Mycobacterium tuberculosis*, since Löwenstein–Jensen medium containing malachite green is the standard medium for the cultivation of small numbers of tubercle bacilli.

Heat resistance of the spores. The spores of the form 2 strains and the *Bacillus licheniformis* strains remained viable after exposure to moist heat at 90° for 20 min., but were not viable after 20 min. at 100°.

Interpretation of the findings

In so far as the results reported here on the properties of form 2 strains confirm those reported by Csillag (1961, 1962, 1963*a*, *b*), no serious differences have been found. All four form 2 strains appear to be of the 'large colony' type which she considered to be the final form of these organisms when obtained from the majority of cultures of *Mycobacterium tuberculosis* (Csillag, 1962). There is agreement that microscopically they appear as rather coarse (0·8 μ thick) rods and filaments, with extremely variable Gram-staining reaction, and form endospores; both accounts of the degree of heat resistance of the latter are consistent (Csillag, 1962). All the form 2 strains grow rapidly on nutrient agar and are facultatively anaerobic. There can be no question therefore that organisms of this kind can be isolated from mycobacterial cultures handled in the way described by Csillag; only the interpretation of the findings is in doubt.

In any experimental situation in which bacterial cultures are found to show altered characteristics, there are two possibilities: either the new properties have been acquired by the parent stock (in which case one is working with a population genetically derived from the parent) or the characters are those of an extraneous and unrelated species, contamination being the explanation of the findings. When a variant can be isolated regularly under defined conditions, when only one or a few of its properties are found to be changed, and when by a considerable number of other tests the new population can be identified with the parent, contamination

need not be considered. The situation is different, however, when several characteristics appear to have altered simultaneously. There are no definite rules about the number of characteristics by which a variant may differ from its parent and still be accepted as such rather than as another species, but in general terms, the greater its divergence from the parent, the greater is the likelihood that another species is being investigated (Hilson & Elek, 1959). When the new population under investigation can be identified with a well-defined (different) bacterial species, especially when the latter is well known to be a common laboratory contaminant, then the probability of contamination is overwhelming. Very weighty experimental evidence to the contrary is needed before the alternative hypothesis of a new and unparalleled genetic change may be accepted.

The four strains of form 2 bacteria studied differ from *Mycobacterium tuberculosis* in many ways, showing characters gained (such as motility, spore formation, facultative anaerobiosis) and lost (such as acid-fastness, urease activity). These properties appear to be genetically determined, since they are stable after serial subculture and after storage. In general terms the mathematical probability of the appearance in a bacterial population of a variant showing multiple genetic changes is the geometric sum of the probabilities for each individual change. It could, however, be argued that the simultaneous appearance of several altered characters might be mediated through a smaller number of actual genetic changes, with linkage of metabolic systems as the explanation of the numerous phenotypic differences; the mathematical probability of an event of this kind taking place would of course be less minute. The nearest analogy is perhaps provided by the appearance of the resistance transfer factor in Shigella species (Nakaya, Nakamura & Murata, 1960; Watanabe & Fukasawa, 1960) which mediates the simultaneous development of resistance to streptomycin, chloramphenicol, tetracycline and sulphonamide. This phenomenon, however, provides only a very remote analogy to the one under discussion; and there is no other evidence in the field of bacterial genetics to indicate that changes in so many unrelated structural and functional characters could be mediated by one, or very few, mutational steps. A large number of independent genetic alterations would be needed, and these could be found only in a bacterial population of astronomical size initially, or after an astronomical number of generations. However, form 2 organisms do not appear in mycobacterial cultures until various periods after the active growth phase is over, when there is little further multiplication. It is therefore highly unlikely on genetic grounds that form 2 bacteria are really the progeny of the mycobacterial population in which they appear.

On the other hand, all four form 2 strains possess in common more than thirty characteristics shown by NCTC strains of *Bacillus licheniformis* tested in parallel, the only difference of note being in respect of penicillin sensitivity, in which the *B. licheniformis* strains themselves were not consistent. Furthermore, all four form 2 strains possessed all the criteria for the identification of the species *B. licheniformis* described by Knight & Proom (1950). Therefore, whether the identity of these strains is determined on the time-honoured basis of giving greater weight to certain properties (e.g. growth rate, endospore formation) than to others, or on the Adansonian basis, more favoured at present, of an assessment of the sum total of properties held in common (Sneath, 1962), the answer is clearly the same: all four are examples of *B. licheniformis*.

This conclusion differs fundamentally from that of Csillag (1961, 1963*a*, *b*), who, though noting the general resemblance between form 2 bacteria and organisms of the genus *Bacillus*, did not classify them in this group but suggested that they should be placed in the Order Actinomycetales, with closest resemblance to the Actinomycetaceae. This would make them more nearly related to the mycobacteria than if they were placed in the genus *Bacillus*. The criteria on which this decision was reached were almost entirely morphological, being based on a study of the macroscopic and microscopic appearances of slide-cultures on glycerol agar sampled at intervals up to 6 weeks; no investigation of metabolic activities was made, apart from a comparison of the effect of aerobiosis and anaerobiosis on the cultures. It should be noted, however, that only two reasons were given for not including these organisms in the genus *Bacillus*: (1) the high incidence of Gram-negative forms; (2) the formation of 'coccoid elements' apparently capable of continued multiplication by binary fission. In fact, in respect of variability of staining, these organisms cannot be distinguished from the NCTC strains of *Bacillus licheniformis* and they are Gram-positive in the taxonomic sense. The significance of the second reason (binary fission of 'coccoid elements') is somewhat obscure. It was assumed that the coccoid elements were formed in the same way as the 'fragmentation spores' of the Actinomycetaceae, and were analogous to them. This assumption was based on observations made of different cultures incubated for various periods, an approach that renders morphological deduction difficult. Leaving aside such difficulties of interpretation, the morphology of species belonging to the genus *Bacillus* and to the Actinomycetales is extremely variable and dependent on the minutiae of medium composition and conditions of culture (Knight & Proom, 1950; Waksman, 1957). No direct comparison of form 2 strains with Bacillus strains on glycerol agar was reported. It is conceivable that coccoid elements might also have appeared in the latter under such conditions. On the other hand, Csillag mentions three characters of the form 2 bacteria which are inconsistent with classification within the Actinomycetaceae: endospore formation, absence of true branching, and lateness of formation of 'coccoid fragmentation spores'. The first of these three is perhaps the most important, since the endospores are heat resistant, a property confined to the spores of the genera *Bacillus* and *Clostridium*.

The phenomenon of 'dimorphism' in fungi was suggested as an analogy to the appearance of form 2 bacteria in mycobacterial cultures (Csillag, 1961). This term dimorphism is applied to the change in morphology which is manifested by certain fungal species in response to a defined environmental stimulus, and which is reversed when the stimulus is removed. The phenomenon is exemplified by the hypha-to-yeast change induced in Histoplasma or Blastomyces by raising the temperature of incubation to 37° (Conant *et al.* 1944), or the similar change induced by the addition of carbon dioxide to anaerobic cultures of Mucor (Bartnicki-Garcia, 1963). However, there does not appear to be any real resemblance between the form 2 phenomenon and dimorphism. No reversion to the original acid-fast form has been described. Nor is there any parallel in dimorphism to the widespread metabolic changes shown by form 2 organisms when compared with the mycobacteria from which they are supposed to be derived. In Mucor, for example, alternation in morphology induced by carbon dioxide is accompanied by little or no change in major physiological processes (Bartnicki-Garcia, 1963).

The evidence presented above shows, first, that the possibility that form 2 bacteria may be genetically derived from mycobacteria must be considered as very remote, and, secondly, that they can be identified as *Bacillus licheniformis*, a spore-bearing species commonly found as a laboratory contaminant. There is therefore a strong *a priori* suspicion that contamination is the explanation of the form 2 phenomenon. In postulating a different origin for these organisms, in the manner of Csillag, the burden of proof lies in showing that they can be isolated regularly from mycobacteria under defined conditions, and that their rate of isolation from mycobacterial cultures is significantly greater than from properly designed controls kept under identical conditions. However, in work to be published in detail elsewhere, I did not find it possible to reproduce the regularity of isolation of form 2 bacteria described by Csillag (1961). I also found that inocula composed of a few spores of form 2 organisms, or of *Bacillus licheniformis* (i.e. inocula of a size which would be expected in random contamination) would not grow on control medium of the kind used by Csillag, whereas they did so readily on the same medium modified by the previous growth of mycobacteria on its surface. The validity of this finding, made by the use of spores freed from vegetative forms by heating, has been contested by Csillag (1964*b*), but my work with unheated spores and with vegetative forms has essentially confirmed it. This being the case, the control cultures of Csillag (1961) were unsatisfactory, since they could not reveal the presence of minimal contamination of this kind, which would, however, be made manifest by the mycobacterial growths in the test cultures. Hence it would appear that the contaminants were derived from the mycobacterial inocula.

It is clear that there is no agreement at present as to the interpretation to be placed upon the form 2 phenomenon, and it is therefore highly desirable that other workers should investigate the problem; however, my belief is that it is unnecessary to postulate a complex life-cycle for mycobacteria, and that the contamination of mycobacterial cultures by extraneous spore-bearing organisms of the genus *Bacillus* provides the entire explanation.

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Inheritance of Capsule and the Manner of Cell-Wall Formation in *Bacillus anthracis*

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SUMMARY

During growth in broth, *Bacillus anthracis* strain 2160s became capsulated only towards the end of exponential growth. An inoculum of fully capsulated organisms formed chains which were non-capsulated save at their tips and at occasional junctions between neighbouring cells. This appearance suggested that new non-capsulated wall was synthesized at the equator of each organism but not at the poles or throughout the existing wall.

INTRODUCTION

Although the synthesis of capsular material by bacteria has been studied extensively, there do not appear to be any reports of the fate of capsules once they have been formed. In many species capsule formation is determined by the growth phase of the culture (Meynell, 1961) and may therefore be suppressed although growth occurs. Thus Meynell & Meynell (1965) found *Bacillus anthracis* was not capsulated until the end of the phase of exponential growth, and the present paper shows that when fully capsulated organisms divide after subculture to fresh broth, their capsules are partitioned amongst their progeny. This is believed to result from the way in which new cell wall is synthesized.

METHODS

Bacteria and culture media. The organism examined is a mutant (D 31) of *Bacillus anthracis* 2160s (Meynell & Meynell, 1964) which differs from its parent by forming capsules in air. It was grown overnight on a shaker in 2 ml. medium containing (g./100 ml.): 'Lab-Lemco', 0·03; Tryptone (Oxoid), 0·07; peptone (Oxoid), 0·3; NaCl, 0·23; dissolved in distilled water and adjusted to pH 7·2. The overnight culture of fully capsulated organisms was diluted 1/15–1/30 in the same medium or in 1% (w/v) acid-hydrolysed casein + 1% (w/v) glucose in 0·05 M-phosphate buffer (pH 7·2), supplemented with tryptophan and adenine (each 20 μg./ml.) and thiamine (2 μg./ml.). Cultures were grown in air at 37° in a 250 ml. conical flask, with either 30 ml. medium with gentle shaking or 5 ml. medium without shaking.

Staining. Most of the films were examined after combined positive and negative staining by carbol fuchsin and nigrosin. Two lines about 1 cm. apart were drawn with grease pencil across a slide and a drop of culture placed between them, mixed with a drop of buffered formaldehyde (40%, w/v, formaldehyde solution diluted 1/40 in 0·05 M-glycerophosphate buffer pH 7·2), and allowed to dry in air. Positive staining was done with Ziehl–Neelsen carbol fuchsin (*Mackie & McCartney's*

Handbook, 1960) freshly diluted 1/10 in distilled water. After 1 min., the film was washed with flowing tap water and gently blotted. One drop of 5% (w/v) nigrosin solution was added and spread over the film by drawing a wire across the drop.The slide was dried by standing it on edge on blotting paper. The nigrosin used was aqueous Nigrosin, batch no. 1062, received in 1962 from George T. Gurr Ltd., 136/140 New King's Road, London, S.W.6. A more recent batch (no. 1552) which was blacker and more viscous decolorized the carbol fuchsin.

Electron microscopy. Organisms were collected by centrifugation and washed in 0·4% (w/v) formaldehyde solution in distilled water. A drop of bacterial suspension was mixed with a drop of dilute nigrosin (batch no. 2062 diluted about 1/4 with distilled water) on a copper grid bearing a Formvar+carbon supporting film. The surplus fluid was drained with filter paper and the grid dried in air. Specimens were examined in a Phillips EM 200 microscope.

RESULTS

Organisms were examined in wet indian ink films, in heat-fixed films stained by M'Fadyean's polychrome methylene blue, and in formaldehyde-fixed films stained by carbol fuchsin+nigrosin. Most of the observations were made by the last method; all three gave similar results. M'Fadyean's stain was never completely satisfactory. The relation between capsule and the individual organisms in a chain was not shown as clearly as in indian ink preparations or by the carbol fuchsin+nigrosin stain, and the red component was found to be unstable in one sample of the stain as it eventually lost the power to stain the capsule at all. In films, the red colour always faded after a few hours under immersion oil. Indian ink films had to be examined immediately because particles of ink soon accumulated in masses on the organisms. The carbol fuchsin+nigrosin stain had none of these disadvantages and was convenient for studies of growth since the immediate fixation of samples by formaldehyde allowed more accurate timing.

The results of electron microscopy after negative staining with nigrosin were qualitatively similar to those observed in the light microscope with stained specimens.

A mixture of fully capsulated and non-capsulated organisms stained by carbol fuchsin+nigrosin is shown in Pl. 1, fig. 1. In the original film, the bodies of the organisms appeared a brilliant red, the capsules were white, and the background had the bluish black colour typical of nigrosin.

Bacillus anthracis 2160s forms chains of 2 to 4 organisms in overnight culture and up to 50 organisms during exponential growth. With an inoculum of fully capsulated organisms like those shown in Pl. 1, figs. 1, 5 the chains formed after incubation for about 3 hr nearly always had blobs of capsule at their ends and at various intermediate points along their length which invariably overlapped the junctions between neighbouring organisms (Pl. 1, figs. 2–4). Occasionally a fully capsulated organism was seen at the end, or less often in the middle, of a chain. In all cases, capsule extended over the union between the fully capsulated organism and its non-capsulated neighbour. Less commonly, a chain had a blob of capsule at one end only (Pl. 1, fig. 6). This was rare 1·5–2 hr after inoculation, but became more common with longer incubation.

DISCUSSION

The most straightforward interpretation of our results is that when capsulated organisms of this strain were subcultured to fresh broth and multiplied in conditions which prevented further capsule formation, their capsules were partitioned amongst their progeny in a manner determined by the way in which new cell-wall was made. A less plausible interpretation is that the capsule was not attached to the bacterium but formed a shell around it which split in half at division, irrespective of how new cell-wall was formed. Various patterns of cell-wall synthesis have been suggested for bacteria. Of the four shown schematically in Fig. 1, one pattern presupposes a

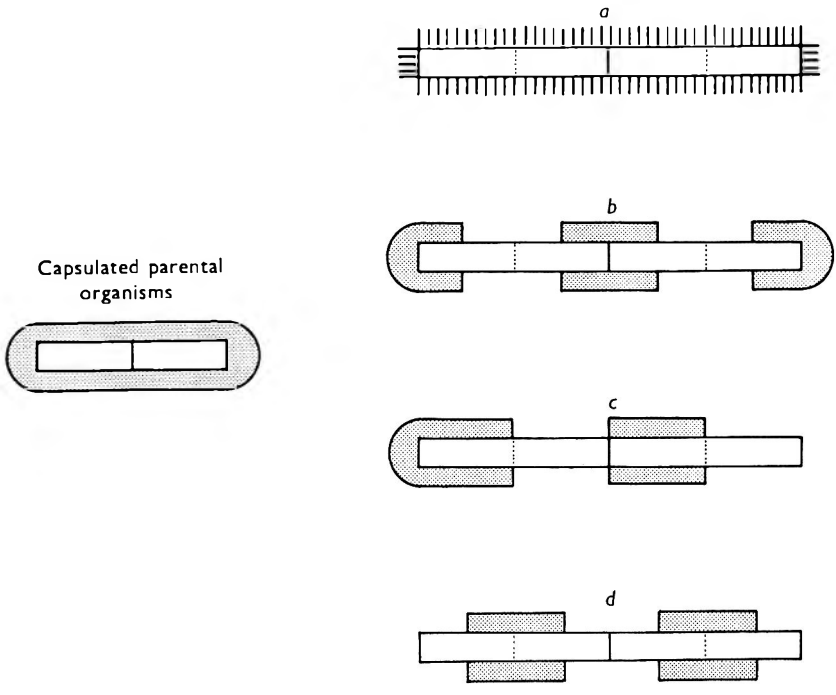

Fig. 1. Cell-wall synthesis and its effect on the inheritance of capsule. A pair of fully capsulated organisms is shown on the left and four possible types of daughters on the right. New transverse septa are shown by dotted lines. Assuming wall and capsule to be associated, diffuse synthesis of new wall leads to (*a*); equatorial growth to (*b*); unipolar growth to (*c*)—two other permutations might occur but all produce daughters that are either non-capsulated or fully capsulated; and bipolar growth leads to (*d*). Only equatorial growth accounts for the appearances shown in Pl. 1.

diffuse process in which new wall appears uniformly amongst pre-existent wall ('intercalation': Quadling & Stocker, 1962; May, 1963), and the other three patterns some form of localized process in which new wall is synthesized at one point in the parent, as in budding, so that new and old wall are sharply separated in the progeny. The anthrax capsule is clearly inherited not diffusely but in blocks, so that cell-wall synthesis by intercalation is excluded if the parental wall and its capsule remain

joined. As regards localized processes, the main possibilities are equatorial growth as in *Streptococcus faecalis* (Chung, Hawirko & Isaac, 1964*b*) and polar growth (Bisset, 1956; Bisset & Pease, 1957). Direct observation of individual organisms shows, however, that new cell-wall is not always formed in precisely the same way at each generation (e.g. fig. 5*c* in Chung *et al.* 1964*b*).

Equatorial growth accounts for the appearance of the chains shown in Pl. 1, figs. 2–4. The inoculum usually consist of pairs of fully capsulated organisms (Pl. 1, fig. 1) whose progeny on division remain linked together. The original cell-wall of the parental organisms and its associated capsule are presumably partitioned between daughters at the first division, as shown in Fig. 1 and continue to be inherited together at later divisions. This will result in the invariable presence of capsule at the extremities of the terminal organisms of the chain and, when the chain originated from n organisms, at $n-1$ points along the chain. At these points the capsule must lie across the junction of neighbouring organisms but never round their equators. This is precisely what was observed. When the capsule was present at one point along the chain, there were often unequal numbers of organisms between it and the ends, presumably because of asynchrony of division. It is to be expected from equatorial growth that half of each terminal organism will be capsulated, on the average, but in fact only about one-fifth had capsule. The reason for this discrepancy is partly due to the fact that the multiplying bacteria were longer than the organisms in the stationary phase, but the residues of capsules may collapse upon themselves or the staining methods may underestimate their size.

Polar growth is incompatible with the observed results, whatever form it takes (see Fig. 1). If unipolar, the process is equivalent to budding and results in daughter organisms with either the full complement of capsule or none at all; if bipolar, capsule is confined to junctions within the chain and is absent from the ends. The occasional chains in young cultures which lacked capsule at one end (Pl. 1, fig. 6) might have arisen in this way, but might equally well have been formed by breakage of chains like those shown in Pl. 1, figs. 2–4, an explanation supported by their increasing frequency as growth continued.

Cell-wall formation can be studied more directly by fluorescence microscopy, using labelled antibody in the method introduced by Cole & Hahn (1962). The growth of *Bacillus anthracis* has not been examined in this way but that of two related species, *B. cereus* and *B. megaterium*, was investigated by Chung, Hawirko & Isaac (1964*a*). In neither of these latter species did intercalation occur; both grew equatorially and *B. megaterium* also grew at the poles (see fig. 2*b* in Chung *et al.* 1964*a*). Photographs of the clones which were increasing by equatorial growth resemble those shown here in Pl. 1, figs. 2–4, with patches of fluorescence which indicated the position of parental cell-wall at the ends of chains and at intermediate points, similar to the distribution of inherited pieces of capsule.

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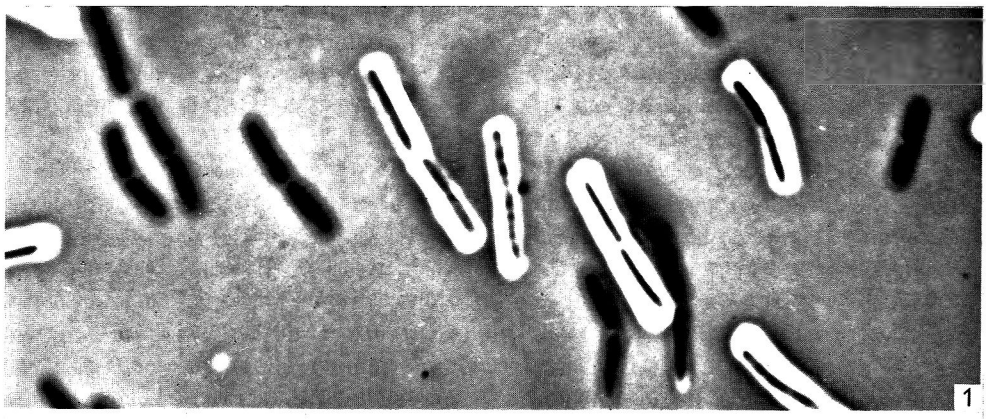
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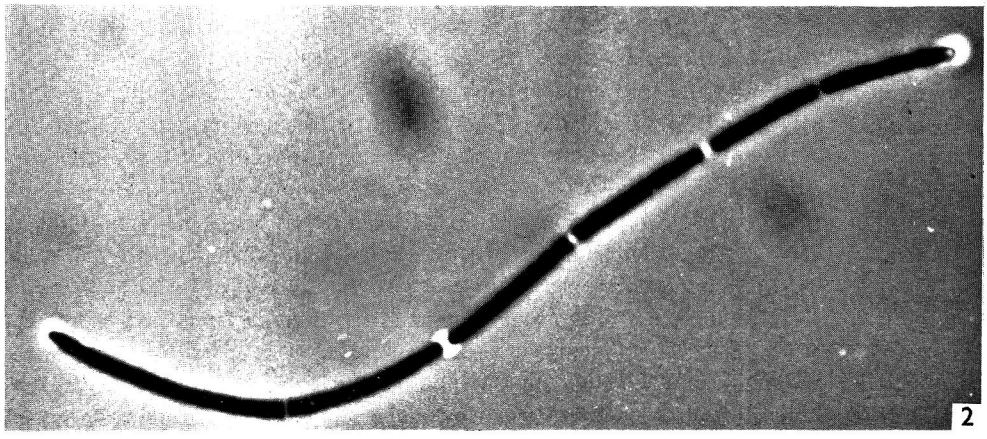
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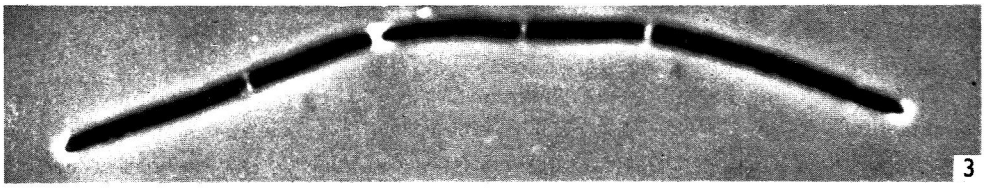
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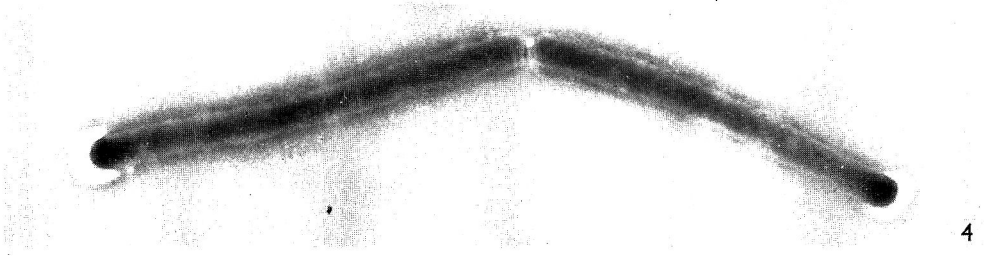
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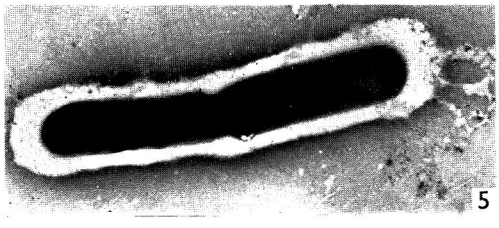
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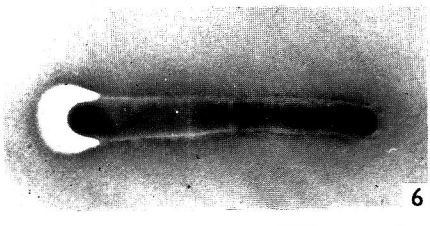
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EXPLANATION OF PLATE 1

Bacillus anthracis: a mutant (D 31) of *B. anthracis* 2160 S (Meynell & Meynell, 1964). The organisms in figs. 2, 3, 4, and 6 came from growing cultures inoculated 1·5 to 3 hr earlier with fully capsulated organisms.

Fig. 1. Fully capsulated organisms from an overnight culture mixed with non-capsulated organisms. Carbol fuchsin + nigrosin.

Figs. 2, 3. Chains with capsules at each end and at one or more points along their length. Carbol fuchsin + nigrosin.

Fig. 4. Chain with capsules at both ends. Nigrosin negative staining. Electron micrograph.

Fig. 5. Capsulated organisms from an overnight culture. Nigrosin negative staining. Electron micrograph.

Fig. 6. A pair of organisms with capsule at one end only. Nigrosin negative staining. Electron micrograph.

J. gen. Microbiol. (1965), **39**, 429

Books Received

Common Colds and Related Diseases. By D. A. J. Tyrrell. Published by Edward Arnold (Publishers) Ltd., 41 Maddox Street, London, W.1. 197 pp. Price 42*s*.

Influenza and other Virus Infections of the Respiratory Tract. By C. H. Stuart-Harris. Published by Edward Arnold (Publishers) Ltd., 41 Maddox Street, London, W.1. 248 pp. Price 45*s*. 2nd edition.

Microbial Inhibitors in Food. Fourth International Symposium on Food Microbiology, 1–5 June 1964, held at the Swedish Institute for Food Preservation Research (SIK), Göteborg, Sweden. Edited by N. Molin. Published by Almqvist & Wiksell, 26 Gamla Brogatan, Stockholm C., Sweden. 402 pp. Price S.Kr. 45.

Neurospora Bibliography and Index. By Barbara J. Bachmann and Walter N. Strickland. Published by Yale University Press, 6*a* Bedford Square, London, W.C.1. 225 pp. 30*s*.

L-forms, Episomes and Auto-Immune Disease. By P. E. Pease. Published by E. & S. Livingstone Ltd., Nos. 15–17, Teviot Place, Edinburgh. 83 pp. Price 10*s*. 6*d*.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-third General Meeting at the Middlesex Hospital Medical School on Monday, Tuesday and Wednesday, 5, 6 *and* 7 *April* 1965. *The following communications were made:*

ORIGINAL PAPERS

The Chromatophores of *Chlorobium thiosulphatophilum*. By J. Sykes and Judith A. Gibbon (*Department of Biochemistry, University of Sheffield*) and D. S. Hoare (*A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield; present address, Department of Microbiology, University of Texas, U.S.A.*).

Earlier reports (Bergeron, J. A. & Fuller, R. C. (1961) in *Biological Structure and Function*, vol. 2, 307, Academic Press) have indicated that the chromatophores from the green, photosynthetic anaerobe *Chlorobium thiosulphatophilum* may be the smallest units capable of photosynthetic activity so far identified; the chromatophores were reported to sediment with the 50*S* component present in the analytical ultracentrifuge diagrams of the crude extracts from this organism, to be nucleic acid free and to have an estimated molecular weight of $1{\cdot}5 \times 10^6$.

Extracts from *Chlorobium thiosulphatophilum* show four major boundaries in the analytical ultracentrifuge, the pigmented chromatophore fraction and three components sedimenting at 47, 30 and $3S_{w,20}$ respectively. The sedimentation of the chromatophore fraction depends strongly upon concentration. Procedures will be described for isolating this fraction in good yield to give an homogeneous preparation sedimenting at $S^0_{w,20} = 116$. A ribonucleoprotein fraction sedimenting with an $S_{w,20} = 47$ may also be isolated from the same crude extract. Chemical and density gradient analyses, together with susceptibility to ribonuclease, further distinguish the pigmented $116S_{w,20}$ material from the $47S_{w,20}$ fraction. The $47S_{w,20}$ component in crude extracts of this organism appears to be a typical bacterial ribosome and is not the pigment carrying component as was earlier reported.

Experiments will also be reported supporting the recent electron-microscope observations with *Chlorobium thiosulphatophilum* (Cohen-Bazire, G., Pfennig, N. & Kunisawa, R. (1964), *J. cell. Biol.* **22**, 207) which indicate that the chromatophores in this organism are true cytoplasmic elements and are not derived by comminution of the cytoplasmic membrane.

The Fine Structure of Uni-cellular Blue Green Algae from the Genus *Merismopedia*. By Dr P. Echlin (*The Botany School, Downing Street, Cambridge*)

There are currently three different species of *Merismopedia* in the Cambridge Collection of Algae and Protozoa; *M. glauca* (CCAP 1448/1), *M. punctata* (CCAP 1448/2) and a species (CCAP 1481/1) variously referred to the genus *Tetrachloris* or *Merismopedia*. An electron microscope investigation has been undertaken to ascertain whether details of their fine structure may help to elucidate the correct taxonomic position of these organisms.

Merismopedia glauca is usually seen as a pair of hemi-cells about 8 μ across. The outer layer of the tripartite cell envelope is highly convoluted and cell division occurs by a typical median constriction in the region of the hemi-cell waist. There appears to be a wide range in the form of the photosynthetic structures. In actively growing cultures the photosynthetic organelles are represented by a series of tightly packed lamellae, in older cultures these structures are replaced by a series of membrane bound spherical and elongate vesicles.

The species of *Merismopedia/Tetrachloris* is a coccus 2–3μ in diameter and does not appear to share the typical cyanophycean characteristics of the previous species. The photosynthetic apparatus is limited to three or four peripherally located lamellae, no variation being seen in older cultures. Dense 'protein bodies' are found associated with the diffuse nucleoplasm.

Merismopedia punctata is a 3–4μ hemi-cell with a structure intermediate between that of the two other forms. Unlike either form, *M. punctata* has a series of filamentous appendages radiating from the outer layer of the cell envelope. These filaments may be associated with the formation and/or attachment of the fibrous sheath layer found in this species.

All three forms are grown and maintained on a soil extract medium, but not all have the emerald-green colour frequently associated with the blue-green algae. It should be noted that the *Merismopedia glauca* and the species of *Tetrachloris/Merismopedia* have lost the regular colonial formation usually associated with the genus. Though colonies of at least sixteen cells have been reported in both strains in past years, no such colonial formation has been observed in the *M. punctata*.

Germination of Protoplast-like Structures from *Fusarium culmorum* Conidia. By J. R. Villanueva, I. Garcia Acha and M. J. R. Aguirre (*Institute de Biologia Celular, C.S.I.C. Madrid*)

Digestion of fungal mycelium by lytic enzyme preparations of actinomycetes has been described (Aguirre, M. J. R., Garcia Acha, I. & Villanueva, J. R. (1964), *Antonie van Leeuwenhoek* **30**, 33) and several instances of lysis of fungi by gut juice of snails are known. Culture filtrates of *Micromonospora chalcea* and *Streptomyces* RA also actively digest the outer layers of the macroconidia of the mould *Fusarium culmorum* (Garcia Acha, I. *et al.* (1964), *Can. J. Microbiol.* **10**, 99). The mode of action of these lytic systems was found to be different and results will be described. The terminal cells of the elongated conidia, the first to germinate under normal conditions, are the more readily attacked by these lytic systems.

Electron microscope studies revealed at least three layers surrounding the cytoplasmic contents of each cell of the spore. After the enzymic treatment, the more rigid layers disappeared, and there remained a wall or membrane which allowed the cells to be completely spherical under stabilized conditions. Protoplasts obtained from conidia were much more stable than those from young hyphae. When these spherical bodies were washed and transferred to a growth medium, they yielded an apparently normal mycelium. Under certain conditions, a kind of pseudomycelium, similar to that of certain yeasts, was formed. Studies are in progress to compare the structure and chemical composition of normal mycelium with the one formed after the enzymic treatment.

Age of spores is critical in these studies. When too young (2–3 days) they are readily digested; when older (more than 20 days) all the content gathers together in the central cells, the attack is slower and results are different. The experiments here described have been obtained when using 5-day-old macroconidia, harvested from a complex medium.

The lytic technique appears very useful for the liberation of protoplasts in *Fusarium culmorum*, and we are trying to extend it to other fungi. Preliminary studies showed differences in the efficiency of strepzyme on conidia of microscopic fungi. Isolated 'protoplasts' survived the lytic enzyme treatment. By using the micromanipulator we have been able to isolate each of the cells of the multicellular conidia which germinated readily.

An Electron Microscopic Study of Nuclear Division in Synchronously Dividing Yeast Cultures. By D. H. Williamson (*John Innes Institute, Bayfordbury, Hertford, Herts.*)

Despite general agreement that the nucleus of *Saccharomyces cerevisiae* is a Feulgen-positive extra-vacuolar body, the fine structure of this organelle is poorly understood, and little is known of its mode of division. The light microscope has yielded conflicting results because the structures involved are difficult to fix and stain, and are near the limit of

resolution. However, in an electron microscopic study, Yotsuyanagi ((1960), *C.R. Acad. Sci., Paris*, **250**, 1522) reported the presence in yeast nuclei of electron-transparent areas containing fibrillar material. Using a staining technique supposedly specific for DNA, he identified these areas as chromosomes, but did not describe their behaviour during nuclear division. In the present studies the disposition of the chromosomes throughout the cell cycle was followed by electron microscopic examination of samples from synchronously dividing cultures. In resting cells the chromosomes aggregate into a mass occupying a small part of the nucleus. Soon after inoculation into fresh medium, they separate, but remain in a loose group on one side of the nucleus until after bud formation and the completion of DNA synthesis. Then, nearly half-way through the cell cycle, the chromosomes segregate, without the aid of a spindle, into two groups. This occurs within the nuclear membrane, which at the same time develops a pronounced equatorial furrow. Immediately thereafter, the nucleus migrates half-way into the bud, carrying one set of chromosomes with it. The nuclear membrane then invaginates, and two nuclei result, the division process being completed about three-quarters of the way through the cell cycle. From these observations and others relating to nuclear synthetic activities, it emerges that the nucleus of this species, though functionally equivalent to that of higher organisms, is organized in a different, and presumably more primitive, manner.

Electron Microscopy of *Micrococcus radiodurans*. By AUDREY M. GLAUERT (*Strangeways Research Laboratory, Cambridge*), R. W. HORNE (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*) and MARGARET J. THORNLEY (*Low Temperature Research Station, Cambridge*)

The fine structure of the radiation-resistant bacterium *Micrococcus radiodurans*, isolated by Anderson, Nordan, Cain, Parrish & Duggan ((1956), *Food Tech., Champaign*, **10**, 575) has been studied by electron microscopy of intact and disrupted cells using thin-sectioning and negative-staining techniques (Thornley, Horne & Glauert (1965), *Archiv. Mikrobiol.*, in press) in an attempt to discover any unsual features which might be correlated with the extremely high resistance to radiation.

The cytoplasm and nuclear structures are normal, but the cell wall and sheath are more complex than any so far described for a bacterium. The surface consists of four distinct layers, each having a characteristic fine structure, and one of these layers has been tentatively identified as that responsible for maintaining the rigidity of the cells. Striations with a periodicity of 175–200 Å are visible in thin sections of this layer, and a pseudo-hexagonal array of dark 'holes' is seen in surface views of negatively stained fragments. It is concluded that this layer is the main structural element of the cell wall of *Micrococcus radiodurans*.

The other three layers of the surface have not yet been clearly located in thin sections; one of these layers has a well-defined pattern of hexagonally arranged subunits, similar to that observed in *Spirillum serpens* by Murray ((1963), *Can. J. Microbiol.* **9**, 381). It is not yet known whether any of these surface layers play a part in the high resistance of *Micrococcus radiodurans* to radiation.

A Pilot Scale Continuous Culture Unit suitable for the Growth of Pathogenic Bacteria. By D. G. MACLENNAN (*Commonwealth Serum Laboratories Commission, Victoria, Australia*) (*present address, Microbiology Department, Queen Elizabeth College, London, W.* 8)

A two-litre pilot scale continuous culture unit has been constructed for the cultivation of pathogenic bacteria and has been instrumented accordingly. The design and construction of the unit will be discussed with particular reference to the following characteristics: (1) Multipoint temperature recording and control. Accuracy of control $\pm 0{\cdot}1^\circ$ C. (2) Automatic pH recording and control using a steam sterilizable glass electrode. Accuracy of control $\pm 0{\cdot}02$ pH units. (3) Continuous measurement and recording of turbidity. (4) Electrical heat sterilization of effluent gas. (5) Automatic foam control. (6) Effective

aeration without stirring using a porous false bottom to the fermentor. (7) Variable speed pumps for sterile flow control. (8) Sampling and other aseptic techniques.

This unit has been operated continuously for periods of up to 6 weeks without breakdown or contamination of the culture. The equipment has been in use for about 2 years for studies on *Escherichia coli* ML 30 and *Brucella abortus* S 19.

Ultraviolet Damage to Bacteria at Low Temperatures. By M J. ASHWOOD-SMITH, R. J. MUNSON and B. A. BRIDGES (*Medical Research Council, Radiobiological Research Unit, Harwell, Berkshire*)

The survival of *Escherichia coli* WP 2 (tryptophan-requiring) from ultraviolet irradiation when suspended in M/15 phosphate buffer (pH 7) has been studied over the temperature range 295° to 4° K. In unfrozen suspensions there was no appreciable change in sensitivity between 295° and 263° K. The sensitivity in the presence of ice progressively increased by a factor of 7 when the temperature was lowered from 268° to 194° K. Between 194° and 77° K the sensitivity decreased to 4 times the sensitivity at 295° K and was not appreciably different at 4° K. No increase in sensitivity was observed at 194° K compared with 295° K when the bacteria were suspended in 80% (v/v) glycerol, which at 194° K forms a non-crystalline glass.

In a strain of *Escherichia coli* WP2 lacking the host cell reactivation system, the slopes of the survival curves at 194° K and 295° K were similar although at the latter temperature the curve had a pronounced shoulder which was not present at 194° K. The sensitivity of bacteriophage T, irradiated in M/15 phosphate buffer (pH 7) was increased by a similar factor (2·4) whether the assay organism (*E. coli* WP2) possessed the host cell reactivation system or not. These results will be discussed in terms of the current concepts of biological damage and repair following ultraviolet irradiation.

The Effect of a Double Shift Temperature Shock on Growing Cells of *Bacillus megaterium*. By G. C. WARE and DIANE ROBERTS (*Department of Bacteriology, Bristol University, Canynge Hall, Bristol* 8)

Synchronous division has been obtained with *Bacillus megaterium* by cooling cultures, growing exponentially at 34°, to 15° for 30 min. and then returning to 34° (Hunter-Szybalska, M., Szybalski, W. & DeLamater, E. D. (1956), *J. Bact.* 71, 17). Using a similar method Bruce (in *Influence of Temperature on Biological Systems*, p. 127, Ronald Press, New York, 1957), was unsuccessful in inducing synchrony in *Escherichia coli*. Our experiments with *B. megaterium* did not result in good synchrony so the system was examined microscopically to study the effects, if any, of cold shock on individual cells.

Microcultures of *Bacillus megaterium* on nutrient agar at 37° were observed by phase-contrast microscopy in a constant temperature room. A suitable group of organisms was selected and the time of division of each cell into two daughter cells noted. Later the cultures were subjected to a cold shock and on return to the original temperature the times of the second division of the selected cells were measured. In this way the effect of several temperatures between 6° and 30° applied for either 22·5 or 45 min. on organisms of various cell ages was determined. The total number of cells observed in this way was over 800.

None of the conditions tested produced efficient synchrony of the subsequent division. The effect of the shock was to delay division more or less equally for cells of any age, the length of the delay being related to the severity of the shock.

The Bactericidal Properties of Cephaloridine ('Ceporin'). By CYNTHIA H. O'CALLAGHAN and MONICA J. MARSHALL (*Glaxo Research Ltd., Sefton Park, Stoke Poges, Bucks. and Greenford, Middlesex*)

The *in vitro* activity of cephaloridine against a wide range of bacterial species has previously been reported (Muggleton, P. W., O'Callaghan, C. H. & Stevens, W. K., 1964, *Brit. med. J.* ii, 1234; Barber, M. & Waterworth, P. M., 1964, *Brit. med. J.* ii, 344). These

workers assessed the minimum inhibitory concentrations of the antibiotic by the tube dilution technique, which recorded the bacteriostatic effect. The present communication is about experiments on the bactericidal activity.

Bactericidal activity was determined at 5, 50 and 500 μg./ml. and also at the minimum inhibitory concentrations for the various organisms tested. Cultures either in the logarithmic phase of growth or in the lag phase were employed as inocula. Viability counts were made at various times after the antibiotic had been added, and the percentage kill was estimated in terms of the initial count.

In general, the minimum bacteriostatic levels of cephaloridine are lower for Gram-positive organisms than for Gram-negative ones. The bactericidal effect, however, was much more rapid in nutrient broth against most Gram-negative organisms (e.g. *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*) than against Gram-positive ones, even at the minimum bacteriostatic level. With *Staphylococcus aureus*, the rate of kill was lower, even though the levels of cephaloridine used were several times the bacteriostatic level. Other experiments showed that the bactericidal action of cephaloridine was undiminished in urine or serum.

Experiments were also carried out in which the effect of adding cephaloridine to growing cultures of *Staphylococcus aureus* and *Escherichia coli* was studied by optical density measurements. The addition of the minimum inhibitory concentration of cephaloridine to *E. coli* during the first 4–5 hr resulted in rapid lysis of the cultures. *Staph. aureus* cultures continued to multiply at a diminished rate for a short time after addition of cephaloridine. The organisms were subsequently killed and partial lysis ensued.

The Antibacterial Activity of Chetomin, Gliotoxin and Sporidesmin. By D. Brewer, Dorothy E. Hannah and A. Taylor (*Atlantic Regional Laboratory, National Research Council, Halifax, Canada*)

Sporidesmin (Ronaldson, J. W., Taylor, A., White, E. P. & Abraham, R. J. (1963), *J. chem. Soc.* p. 3172) changes membrane transport properties in hepatic cells (Mortimer, P. H., Taylor, A. & Shorland, F. B. (1962), *Nature, Lond.* **194**, 550), in mitochondria (Gallagher, C. H. (1964), *Biochem. Pharmacol.* **13**, 1017) and in epithelial cells in culture (Done, J., Mortimer, P. H., Taylor, A. & Russell, D. W. (1961), *J. gen. Microbiol.* **26**, 207). Its structure (Hodges, R., Ronaldson, J. W., Taylor, A. & White, E. P. (1963), *Chem. & Ind.* p. 42) relates it to two other groups of microbial metabolic products, the gliotoxins (Herrmann, H., Hodges, R. & Taylor, A. (1964), *J. chem. Soc.* p. 4315) and the chetomins (Waksman, S. A. & Bugie, E. (1944), *J. Bact.* **48**, 527; Brewer, D., Taylor, A. & Vining, L. C., unpublished work). A biological assay procedure has been developed that enables the biological properties of these metabolites to be compared. The concentrations required to induce a fourfold increase in lag phase of 10^6–10^7 *Bacillus subtilis* (ATCC 6633) organisms/ml. growing on Bacto-Penassay broth (Difco) have been measured. Sporidesmin-B was effective at a concentration of 400 μg./ml., sporidesmin at 80 μg./ml., gliotoxin at 2 μg./ml. and one of the chetomin group of metabolites at 0·08 μg./ml. Ten other chemical transformation products and metabolites having the disulphide bridged dioxopyrazine ring system were active within this range. Degradation products and metabolites differing from their parents only in the absence of sulphur were inactive. The increase in lag phase was proportional to the disulphide concentration at low dose levels and was dependent on the inoculum size. The rate of growth after the organisms had entered the logarithmic phase was independent of the concentrations of gliotoxin and chetomin but was dependent on the concentration of sporidesmin.

Adaptation and De-adaptation to Aromatic Substances by Yeasts. By J. S. Hough and D. A. J. Wase (*Department of Biochemistry, University of Birmingham*)

Selected strains of yeast utilize aromatic compounds for growth (Harris, G. & Ricketts, R. W. (1962), *Nature, Lond.* **195**, 473) and these observations have been extended by a sequential induction technique used previously by Stanier ((1947), *J. Bact.* **54**, 339) for

the breakdown of aromatic compounds by bacteria. When yeast cells grown on dilute phenol medium were presented with various aromatic compounds, they were shown to be adapted only if cultures were harvested in the log phase of growth and provided with both a nitrogen source and growth factors.

With yeast grown in glucose medium, adaptation to catechol, phenol, resorcinol and quinol occurred after various lag periods, but there was response to neither protocatechuate nor *p*-hydroxybenzoate. A comparison of lag periods required for adaptation to the various substrates indicates that phenol is converted to catechol before fission, while metabolism of resorcinol and quinol is achieved by separate and differing routes. Cells fully adapted to phenol were simultaneously adapted to quinol, resorcinol and catechol, and also metabolized protocatechuate and *p*-hydroxybenzoate slowly.

In contrast, cells adapted to protocatechuate metabolized catechol, phenol, quinol and resorcinol only after a lag period, and attacked protocatechuate and *p*-hydroxybenzoate slowly. Clearly yeasts present a more complicated response to aromatic compounds than bacteria. This is again manifest by yeast cells adapted to *p*-hydroxybenzoate, which prove to be simultaneously adapted to protocatechuate, catechol and phenol.

The results indicate that any simple aromatic compound may act as a partial inducer for similar compounds, and that at least four separate pathways may be involved in their breakdown.

Influence of Temperature on the Macromolecular Composition of C(Glycerol)-Limited and Mg^{2+}-Limited *Aerobacter aerogenes*, Growing in a Chemostat. By D. W. TEMPEST and J. R. HUNTER (*Microbiological Research Establishment, Porton, Salisbury, Wilts.*)

At a fixed temperature, the RNA content of both C(glycerol)-limited and Mg^{2+}-limited *Aerobacter aerogenes* organisms was a function of growth rate. However, whereas in the C-limited system the *culture* RNA content also varied with growth rate, such was not observed when Mg^{2+} was made the growth limiting component of the medium. Instead an increase in the cellular RNA level was balanced by a reduction in bacterial concentration and the *culture* RNA content remained constant, irrespective of growth rate.

Schaechter, M., Maaloe, O. & Kjeldgaard, N. O. ((1958), *J. gen. Microbiol.* **19**, 592) reported that when the growth rate of *Salmonella typhimurium* cultures was varied by changing incubation temperature, the organisms were not altered with respect to RNA content. In the chemostat, temperature can be varied *whilst maintaining growth rate at a constant value* (i.e. operating at a dilution rate well removed from the 'critical' value). Lowering the temperature of *Aerobacter aerogenes* cultures produced a pattern of changes in macromolecular composition similar to those associated with an increased growth rate. This was particularly evident in the Mg^{2+}-limited system where stoichiometry between *culture* RNA and Mg^{2+} was maintained and a temperature shift resulted in a marked change in growth yield.

These data are compatible with the hypothesis that in growing organisms the ribosomes function at a maximal rate, adjustment of the rate of protein synthesis being effected through a variation in ribosomal concentration. Raising or lowering the ribosomal activity (by varying temperature) results in a change in ribosomal content if the growth rate is maintained constant, or in growth rate if the culture is 'unlimited'.

The Regulation of the Synthesis of Structural Components in the Cell of *Bacillus megaterium*. By J. CHALOUPKA and M. VÁVROVÁ (*Institute of Microbiology, Czechoslovak Academy of Science, Prague*)

The rate of synthesis of the protein portion of the cytoplasmic membrane, the ribosomes, the nuclear equivalents and the cell wall mucopeptide was measured under different physiological conditions. After the transfer of a culture of *Bacillus megaterium* growing logarithmically in an amino acid medium (gen. time 45 min.) to a minimal medium with nitrate as the sole source of nitrogen (gen. time 110 min.), the rate of protein synthesis

decreased to 10–15 % and the mucopeptide synthesis increases by 30–50 %. The incorporation of [^{14}C]adenine into RNA was inhibited in 'step-down' cultures by 90 % or more. The organisms from a step-down experiment which had been labelled by a pulse of [^{14}C]leucine in the minimal medium were converted to protoplasts and further fractionated by ultracentrifugation and ultrasonic disintegration after disruption of the cytoplasmic membrane by detergent. The distribution of radioactivity in different cell fractions was compared with similar fractions obtained from cells labelled under balanced growth conditions. In the organisms from the 'step-down' experiment the respective rates of synthesis of cytoplasmic and membrane protein were the same, the incorporation into ribosomal protein was 40–50 % of normal, and that into 'nuclear' proteins was a little enhanced or the same as that of cytoplasmic proteins. Almost the same results were obtained when a growing culture was transferred into nitrogen-free medium. In 'step-up' organisms, a stimulation of the synthesis of ribosomal protein was found in comparison with the other components. The life time of messenger RNA for different fractions was measured in actinomycin D-treated cultures. The stability of messenger RNA for ribosomal, membrane and cytoplasmic protein does not differ substantially.

Biosynthesis of the Polypeptide Capsule and the Cell Wall of *Bacillus anthracis*
By G. G. Meynell (*Guinness–Lister Research Unit, Lister Institute of Preventive Medicine, London, S.W.* 1) and Elinor Meynell (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 11)

The capsule of the anthrax bacillus consists of polypeptide containing only polymerized D-glutamic acid (PGA). Microscopy shows that it is not formed during exponential growth of strain 2160s, which requires added HCO_3^- for capsule formation (Meynell, E. W. & Meynell, G. G. (1964), *J. gen. Microbiol.* **34**, 153), nor of mutants which differ in forming capsules in air. This adds further support to the view that these mutants differ from their parent only in respect of their HCO_3^- requirement for capsule formation (Meynell, G. G. & Meynell, E. W. (1964), *Proc. Soc. gen. Microbiol.* **37**, i). When non-capsulated cells from mutant cultures in late exponential growth are exposed to either tetracycline (30 μg./ml.) or chloramphenicol (250 μg./ml.), growth stops but capsules appear. This suggests that PGA, like the polypeptide antibiotics gramicidin and tyrocidin, is not synthesized like protein by the assembly of activated amino acids on ribosomes (Okuda, K., Edwards, G. C. & Winnick, T. (1963), *J. Bact.* **85**, 329; Mach, B., Reich, E. & Tatum, E. L. (1963), *Proc. Nat. Acad. Sci., Wash.* **50**, 175). Capsulation as well as growth is inhibited by tetracycline or chloramphenicol if the culture is in early exponential growth, suggesting that the PGA-synthesizing pathway is not in operation until the culture reaches the end of the exponential phase. The organisms in a given culture differ grossly in their ability to form capsules in the presence of the antibiotics (or of adenine deficiency) since each chain usually contains non-capsulated and fully capsulated cells, whose capsules are often abnormally large.

The cell wall grows by the insertion of new material at the equator of each cell, since fully capsulated cells subcultured to fresh medium give rise to chains with remnants of capsule at their extremities and over occasional intercellular junctions. This excludes cell-wall synthesis by a diffuse process or by bipolar or unipolar growth.

Loss of Ability to Concentrate Proline in *Escherichia coli*: Basis of Resistance to Proline Analogues.
By Stella Neale* and H. Tristram (*Department of Botany, University College London, W.C.* 1)

The proline analogues 3,4-dehydro-DL-proline and L-azetidine-2-carboxylic acid strongly inhibit growth of *Escherichia coli*. Both compounds are incorporated into proteins of the organism, stoichiometrically replacing proline (Fowden, L. & Richmond, M. H. (1963), *Biochim. Biophys. Acta*, **71**, 459; Fowden, L., Neale, S. & Tristram, H. (1963), *Nature, Lond.* **199**, 35).

A number of analogue-resistant strains were isolated by plating *Escherichia coli* C 4 on

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glucose-mineral salts agar containing either 3,4-dehydro-DL-proline (25 μg./ml.) or L-azetidine-2-carboxylic acid (25 μg./ml.). All displayed cross-resistance to both analogues and failed to incorporate either dehydroproline or azetidine into cell protein.

A specific transport system permitting the accumulation of exogenous proline by *Escherichia coli* has been described (Britten, R. J. & McClure, F. T. (1962), *Bact. Rev.* **26**, 292). Strain c 4 suspended in glucose-mineral salts medium containing chloramphenicol (150 μg./ml.) at 10° C. rapidly accumulated L-^{14}C-proline in the soluble pool until equilibrium between internal and external concentration was achieved. The simultaneous addition of dehydroproline, azetidine or unlabelled proline competitively inhibited the rate of ^{14}C-proline uptake. When unlabelled proline, dehydroproline or azetidine was added to cells which had previously accumulated ^{14}C-proline, radioactivity was displaced from the pool. Structurally unrelated amino acids and the D-isomers of azetidine or proline were without effect on the levels of ^{14}C-proline accumulated. At 0° C proline accumulation was prevented, but exchange between internal and external proline occurred (Britten, R. J. & McClure, F. T. *loc. cit.*), as did exchange between accumulated ^{14}C-proline and exogenous dehydroproline or azetidine.

All resistant strains so far tested lacked the ability to accumulate proline to high levels. It is concluded that the proline transport system is normally responsible for entry of dehydroproline and azetidine and that the resistant strains possess defective transport systems, thereby excluding the analogues from the cell and consequently preventing their incorporation into protein.

C_2 Mutants of *Pseudomonas aeruginosa*. By A. J. Skinner and Patricia H. Clarke (*Biochemistry Department, University College London*)

We have obtained mutants of *Pseudomonas aeruginosa* 8602, which are unable to grow on acetamide, by treating cultures with either ethyl methane sulphonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The mutants could be divided into several groups on the basis of growth and enzyme studies. Ten mutants grew on acetate but not acetamide. All these lacked the amidase described by Kelly, M. & Clarke, P. H. ((1962), *J. gen. Microbiol.* **27**, 305). Growth in succinate medium plus *N*-acetylacetamide induces amidase synthesis in the wild-type (Brammar, W. J. & Clarke, P. H. (1964), *J. gen. Microbiol.* **37**, 307) but no activity could be detected in any mutants of this group.

The other mutants did not grow on either acetamide or acetate, but amidase could be induced to the normal level. Growth of *Pseudomonas aeruginosa* on acetate as sole carbon source requires an actively functioning glyoxylate cycle (Kornberg, H. L. & Krebs, H. A. (1957), *Nature, Lond.* **179**, 988). One of the essential enzymes is *iso*citrate lyase catalysing the production of glyoxylate and succinate from *iso*citrate. Neither the wild-type nor the mutants grew on glyoxylate alone, but eight of the acetate mutants grew well on acetate + glyoxylate. Extracts of succinate-grown cells were assayed for *iso*citrate lyase (Dixon, G. H. & Kornberg, H. L. (1959), *Biochem. J.* **72**, 3P) and the enzyme was found to be either completely absent or the specific activity was much lower than that of the wild type under the same conditions. The wild type strain grows on propionate and all but two of the acetate mutants grew on propionate but not acetate. The enzyme defects of these two mutants and other acetate mutants are under investigation.

Genetic transfer in *Pseudomonas aeruginosa* can be carried out by phage-mediated transduction. Phage F 116 isolated by Holloway, B. W., Egan, J. B. & Monk, M. ((1960), *Aust. J. exp. Biol.* **38**, 321) was found to transduce amino acid auxotrophs (e.g. His$^-$) of strain 8602. Both the amidase deficient and *iso*citrate lyase deficient mutants can also be transduced by phage F 116.

The Adaptive Hydrolysis of Amides by *Mycobacterium smegmatis* NCTC 8159. By P. Draper (*Department of Biochemistry, University College London*)

Mycobacteria hydrolyse many amides, and the study of their particular substrate specificities has been pursued as a means of classification (Bönicke, R. (1960), *Zbl. Bakt.* (1. *Orig.*) **179**, 209; Nagayama, H., Konno, K. & Oka, S. (1961), *Nature, Lond.* **190**, 1219).

Mycobacterium smegmatis NCTC 8159 grows well on acetamide as sole carbon source, and it was thought interesting to compare it with *Pseudomonas aeruginosa* NCTC 8602 which forms an inducible aliphatic amidase (Kelly, M. & Clarke, P. H. (1962), *J. gen. Microbiol.* **27**, 305). Cells or cell-free extracts of *M. smegmatis*, grown on acetamide, hydrolysed each of 14 amides more than ten times faster than those grown on succinate. *N*-Methyl- and *N*-dimethyl-formamide and *N*-methyl and *N*-dimethylacetamide were not substrates. Formamide was hydrolysed very rapidly by acetamide-grown bacteria, at 800 μM/hr/mg. dry weight, and butyramide at 26 μM/hr/mg. dry weight. Rates for other aliphatic amides were in the order: acetamide < propionamide < *n*-butyramide > *n*-valeramide > *n*-hexoamide $\doteq$ *iso*-butyramide. Bacteria grown on acetate hydrolysed these amides at nearly the same rates as acetamide-grown bacteria; the amidase of *P. aeruginosa* is also induced during growth on acetate (Kelly, M. & Kornberg, H. (1962), *Biochim. biophys. Acta*, **59**, 517). Amidase activity of *M. smegmatis* was not induced by growth on propionate or butyrate, and only slightly on butyramide, though the organism grew well in each case. Cell-free extracts from adapted cells formed hydroxamates from amides and hydroxylamine at rates: acetamide < propionamide > butyramide > valeramide. Formamide did not react. Rates of acyl-transfer to hydroxylamine were much lower than rates of hydrolysis of the amides, whereas for the enzyme from *P. aeruginosa* these rates are roughly similar. *M. smegmatis* hydrolyses a wider range of amides than *P. aeruginosa*, though there is preliminary evidence that the mycobacterial formamidase activity is separately inducible from the enzyme which hydrolyses other alkyl amides.

The Effect of the Presence of an Episome in the Recipient on the Establishment of Transduced Colicinogenic Factors. By P. FREDERICQ (*University of Liège, Belgium*)

The wild colicinogenic strain *Escherichia coli* K 260 is carrying *colV* and *colB* linked to a determinant of fertility. Many recombinants obtained in crosses with *F*− strains, carrying a deletion in the *try* region, are partial diploids, heterozygous for that region. They are of the *F′* type, carrying an episome in which all the genes from the *try* region, including *cysB* and T_1, are linked to an *F* agent and to *colB* and *colV*. In transduction experiments with phage P_1 grown on a donor carrying that episome, about 2% of the *try*+ transductants are colicinogenic, most of them receiving *colV* alone, a few *colB* alone and very exceptionally (less than 1 in 1000) *colV* and *colB* together, at least when the recipient is not already carrying another episome.

When the recipient is already carrying some other episomes, the results are strikingly different. About 50% or more of the *try*+ transductants receive also colicinogeny, this time mostly *colB* alone but quite a number *colV* alone. This effect is observed with the following episomes: three different *F′-lac*, 1 *F′-gal*, two different *Fd* (defective *F* agents) and two different *colV* factors, but not with a normal *F* agent, prophage ϕ80, *col* factors E_1, E_2 or *I*, and two different *R* factors of multiple drug resistance. As the different responses are also observed when a same phage preparation is used, colicinogenic factors must be transmitted to the same extent in all cases but need the presence of an episome to be permanently established and transferred to the progeny of the recipient cell. There is indeed some indication that the transduced *col, try* episome does recombine with the episome already present in the recipient.

Bacteriophage λ: Antigenic Differences between the Wild-Type and Clear Plaque-Forming Mutants. By B. A. FRY and W. M. WAITES (*Department of Microbiology, University of Sheffield*)

The clear plaque-forming mutants, phage λ_v (virulent inducer mutant) and phage λ_c, both isolated from the wild-type phage λ_{22}, are neutralized by antiserum to phage λ_{22} at a slower rate than phage λ_{22} itself. Neutralization constants (*K*) for the antiserum were determined in standardized conditions (Burnet, F. M., Keogh, E. V. & Lush, D. (1937), *Aust. J. exptl. Biol. med. Sci.* 15, 227). For the anti-wild-type serum, *K* for phage λ_{22} was

109 min^{-1}, whereas for phage λ_v it was 27 min^{-1}. On the other hand, the antiserum to the virulent inducer mutant neutralized all three phages at similar rates (K = 84, 76 and 70 min^{-1} for phage λ_v, λ_c and λ_{22} respectively). To check whether the λ_v mutant was typical, fifty independent λ_v-type mutants were isolated from phage λ_{22} and tested against anti-wild-type serum. Forty-two of these mutants gave K values with a mean of 28 min^{-1}: the mean value for the serum for the wild-type was 96 min^{-1}. Three mutants gave a mean K value (93) equivalent to that for the wild-type and appeared to be heterozygous. When plated on a non-lysogenic indicator strain the plaques were at first turbid with one or two clear areas but later became clear all over and typical of those produced by phage λ_v.

Whether there is any connexion between these differences in antigenic behaviour and differences in the ability to lysogenize *Escherichia coli* has yet to be established. These studies with phage λ appear to be the first instance of antigenic differences between the wild type and mutants derived from it. In previous work, e.g. with r mutants and host range mutants of phage T_2 (Hershey, A. D. (1946), *Genetics*, **31**, 620) no significant differences have been reported.

A Haemagglutinin from Coxsackie A7 Virus-infected Tissues. By J. D. Williamson (*Viral Epidemiology Unit, University of Glasgow*)

Haemagglutinins have been shown to be present in extracts of tissues from suckling mice infected with Coxsackie A 7 virus. Haemagglutination was demonstrable with vaccinia-agglutinable fowl red cells but not with human, mouse or vaccinia-inagglutinable fowl cells. Similar extracts of uninfected suckling mice or suckling mice infected with other Coxsackie A viruses did not contain detectable haemagglutinins. Haemagglutination was specifically inhibited by homologous antisera. Haemagglutinating activity was removed from extracts of infected tissues without decrease in infectivity by absorption with vaccinia-agglutinable fowl red cells. In centrifugation studies, haemagglutinating activity sedimented at a greater rate than both infectivity and CF activity.

Preliminary chemical tests have shown that the haemagglutinin is probably a protein and that it resembles other enterovirus haemagglutinins in being inhibited by *p*-chloromercuribenzoic acid. Inhibition by *p*-chloro-mercuribenzoic acid is reversible by treatment with sulphydryl compounds. The fowl red cell receptor for the Coxsackie A 7 haemagglutinin is destroyed by periodate but not by *Vibria cholera* filtrate. Receptors for vaccinia haemagglutinin are also destroyed by periodate, but, unlike Coxsackie A 7 haemagglutinin, these receptors are also destroyed by papain and α-chymotrypsin.

The properties of the Coxsackie A 7 haemagglutinin compared with those of previously described viral haemagglutinins will be discussed.

Assay of Neutralizing Antibodies by Quantitative Haemadsorption. By N. B. Finter (*Research Department, I.C.I. Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

The Quantitative Haemadsorption (QH) technique described for the assay of interferon (Finter, N. B. (1964), *Virology*, **24**, 589) can also be used for assaying neutralizing antibodies to haemadsorbing viruses. Tube tissue cultures of appropriate cells are inoculated with sufficient virus so that, when haemadsorption is carried out after 18–24 hr, the cell sheets are covered with red cells. Surplus red cells are washed away, and the haemadsorbed cells are lysed by adding distilled water. The resultant solutions of haemoglobin are estimated spectrophotometrically at 410 mμ. To measure antibodies, appropriate serum dilutions are incubated with aliquots of virus for 1½ hr at room temperature before adding to groups of tissue culture tubes. As the amount of free virus is reduced by neutralizing antibodies, there is less haemadsorption and weaker haemoglobin solutions are obtained. A plot of the optical densities of the haemoglobin solutions against the log serum dilutions gives an S-shaped curve with an intermediate linear portion from which the 50 % serum neutralizing titre can be derived.

Compared with conventional neutralizing assays, the method has advantages of speed, sensitivity and accuracy. Results are obtained within 24 hr. The sensitivity can be made very high if a small dose of virus is used. Accuracy depends on the number of tubes used to test each serum dilution and on the log dilution steps chosen. The method may be of value for investigating serological relations between haemadsorbing viruses, and for measuring antibodies to those viruses, e.g. certain A 2 influenza viruses, where haemagglutination-inhibition techniques are not satisfactory because of the effects of non-specific inhibitors.

The Interaction Between Riley's Plasma Enzyme Elevating Virus and the Reticuloendothelial System. By K. E. K. ROWSON and B. W. J. MAHY (*Department of Cancer Research, The London Hospital Medical College, London, E.* 1)

Viraemia and an increase in the level of certain plasma enzymes are characteristic features of Riley virus-infected mice. Because some intravenously injected enzymes are cleared from the circulation at a reduced rate in these mice it has been suggested that the raised enzyme levels are due to impaired function of the reticuloendothelial system (RES) (Mahy, B. W. J. (1964), *Virology*, **24**, 481; Notkins, A. L. & Scheele, C. (1964), *J. nat. Cancer Inst.* **33**, 741). This could result from a specific action of the virus on RES cells or be a non-specific effect due to blocking of the RES by prolonged phagocytosis of circulating virus. The viraemia reaches a peak (10^9 to 10^{10} ID 50 per ml. of blood) 18–24 hr after infection and then falls within 15 days to a level of 10^5 ID 50 per ml. of blood, where it remains relatively stable for many months.

Neither stimulation of the RES with stilboestrol nor blockade with thorotrast or zymosan had any effect on virus replication as judged by the level of viraemia attained 15 hr after infection. However, the stable viraemia 10 days or more after Riley virus infection rose promptly after an injection of thorotrast or zymosan, both of which were found to block phagocytosis at this time. Thorotrast has been reported to increase the level of viraemia in Semliki forest virus-infected mice (Mims, C. A. (1964), *Bact. Rev.* **28**, 30).

Contrary to expectation, stimulation of the RES with bacterial endotoxin did not significantly reduce the stable level of viraemia.

Although the clearance rate of enzymes from the blood is reduced in Riley virus-infected mice, preliminary results suggest that the clearance of intravenously injected Riley virus and bacteriophage T 2 is not blocked, and may even be at a slightly faster rate than in normal mice.

Mouse Sarcoma Virus (MSV) Viraemia in the Adult Rat. By A. V. GILLESPIE (*Cancer Research Department, London Hospital Medical College, London, E.* 1)

An apparently new virus which causes the production of tumours in mice, and in newborn rats and hamsters, has been described by Harvey (1964, *Nature, Lond.* **204**, 1104). When newborn Chester Beatty albino rats were injected with virus a viraemia developed which lasted for at least 54 days.

In the present work adult Chester Beatty Hooded rats were injected with MSV and the course of the viraemia followed. The virus first became demonstrable in the plasma from about the fourth day, and disappeared on or about the tenth day, after injection.

The results of neutralization tests suggest that the disappearance of the viraemia is due to the production of neutralizing antibody.

Mutational Immunity to a Colicin. By R. C. CLOWES and E. E. M. MOODY (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

The loss of sensitivity to the lethal action of a colicin by a bacterial strain is usually due to the loss by mutation of an adsorbing receptor, as is also found in phage-resistant mutants. We have recently isolated mutants of *Escherichia coli* K12 which have become

indifferent to the lethal action of a colicin, but which retain the ability to adsorb it. These mutants therefore behave in a similar way to colicin-producing strains which adsorb but are immune to their own colicin.

From colicin E1, all such mutants (comprising some 25 % of all E1-indifferent mutants) become concomitantly hyper-sensitive to a large range of oxidation-reduction dyes including methylene blue and tetrazolium. When dye-resistant revertants are selected they invariably regain sensitivity to colicin. The mutationally immune strains appear to adsorb dye normally, but are unable to reduce it. The dyes are highly bactericidal and within a few minutes of exposure less than 1 in 10^6 survivors are found.

A genetic locus conferring E1-immunity/dye sensitivity has been mapped. Complementation studies observing transfer by Hfr strains of either the wild-type locus (dye resistance) or mutant locus (E1-immunity), either of which can be selected, suggest that the mutation leads to production of a defective enzyme.

Nutritional studies of immune mutants suggest that this defect involves the enzyme succinic dehydrogenase. Since this enzyme appears to act at the level of flavoprotein, and is involved in the coupling of phosphorylation with the electron transport system (also the level of action of many of the dyes), these results are in line with the recent suggestion (Levinthal, quoted by Luria (1964), *Ann. Inst. Pasteur*, **107**, suppl. to no. 5, 65) that colicin E1 acts by stopping energy flow from oxidative phosphorylation.

SYMPOSIUM ON STRUCTURE AND FUNCTION IN MICRO-ORGANISMS

Two days (6 and 7 April) were given to a Symposium on 'Function and Structure in Micro-organisms'. The principal contributions were published as a book, the fifteenth of the Society's Symposia. The titles of the contributions are given below:

Introduction. The Architecture of the Microbial Cell. By the late D. D. Woods, (*Department of Biochemistry, University of Oxford*).

Comparative Aspects of Structures Associated with Electron Transport. By June Lascelles (*Microbiology Unit, Department of Biochemistry, University of Oxford*).

The Concentration of Small Molecules Within the Microbial Cell. By R. J. Britten (*Carnegie Institute of Washington, Washington, D.C., U.S.A.*).

Passage of Particles and Macromolecules Through Cellular Membranes. By H. Holter (*Carlsberg Laboratories, Physiological Department, Copenhagen*).

Secretion of Enzymes by Micro-organisms. By J. O. Lampen (*Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J., U.S.A.*).

The Physical Organization of Nucleic Acid and Protein Synthesis. By K. McQuillen (*Sub-department of Chemical Microbiology, Dept. of Biochemistry, University of Cambridge*).

The Biosynthesis of Nucleic Acids and their Role in Protein Synthesis. By K. A. Stacey (*M.R.C. Microbial Genetics Research Unit, Ducane Road, London, W.*12).

The Outer Layers of Bacteria—The Biosynthesis of Structure. By H. J. Rogers (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

Flagellar and Ciliary Movement in Micro-organisms. By B. A. Newton and D. Kerridge (*Sub-department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*).

Hydrodynamic Aspects of Microbial Movement. By R. E. Burge and M. E. J. Holwill (*Department of Physics, Queen Elizabeth College, London, W.*8).

Cytoplasmic Streaming and Amoeboid Movement. By L. WOLPERT (*Department of Zoology, King's College, London, W.C.*2).

Structure and Function of the Bacterial Chromosome. By W. HAYES (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.*12).

On Non-chromosomal Heredity in Micro-organisms. By RUTH SAGER (*Columbia University, New York, N.Y., U.S.A.*).

Sequential Expression of Biochemical Events During Intracellular Differentiation. By H. O. HALVORSON (*Department of Bacteriology, University of Wisconsin, Madison* 6, *Wisconsin, U.S.A.*).

A Consideration of Bacterial Membrane as the Agent of Differentiation. By P. FITZ-JAMES (*Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario*).

The Partitioning of Genomes. By D. MAZIA (*Department of Zoology, University of California, Berkeley, California, U.S.A.*).

Index of Authors

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology

Index of Subjects

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology